



Studies on *Arcobacter* species, their isolation and pathogenicity.

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Abstract

Arcobacter species, a genus previously classified as ‘aerotolerant campylobacters’, have gained consideration as emerging food and water-borne pathogens. Studies increasingly suggest that they are of significance to veterinary public health and agriculture, but their pathogenicity mechanisms and ability to cause disease in animals and humans are not well understood.

This project has revealed that only two of eleven Scottish surface water sources were contaminated with *Arcobacter* (18 %), and this was the first isolation of *Arcobacter* spp. from surface waters in Scotland. The two *Arcobacter* water isolates (SW-DL2 and SW-OL2) were shown to be closely related to species of *Arcobacter butzleri*. The extracellular proteins (ECP) of all strains were positive for gelatinase activity (50-310 units) and caused haemolysis of sheep and chicken erythrocytes. The *A. butzleri* reference strain D2686 showed poor haemolysis of human erythrocytes. The *cadF* genes of *A. butzleri* D2686 and SW-OL2 were cloned and sequenced, and showed high levels of sequence identity with a gene that encodes fibronectin-binding protein in *Campylobacter jejuni*. All *Arcobacter* strains in this study showed the ability to adhere to INT-407 cells *in vitro* and mutagenizing the *cadF* genes of *A. butzleri* D2686 and SW-OL2 resulted in a significant reduction in adherence ($P > 0.01$ and $P > 0.001$ respectively). Five putative virulence genes (*cadF*, *ciaB*, *flaA*, *flaB* and *pldA*) have been detected in each of the *Arcobacter* strains studied. *cadF* mRNA was also shown to be expressed in all strains with higher levels in *A. cryaerophilus* and SW-OL2 than the other strains. RT-PCR analysis also revealed that expression of the *cadF* gene was upregulated in all strains on infection of tissue culture cells with significant differences in levels of expression observed.

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Abbreviations

APS	ammonium persulfate
°C	degrees centigrade
bp	base pairs
ddH₂O	double distilled water
g	gram
× g	gravity force
hr	hours
k	kilo
kD	kilodalton
µg	microgram
mg	milligram
min	minute
mm	millimetre
µl	microlitre
ml	millilitre
pmol	picomole
mM	millimolar
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TEMED	Tetramethylethylenediamine
V	voltage
w/v	weight per volume
EMJH	Ellinghausen-McCullough-Johnson-Harris

CHAPTER 1 INTRODUCTION

1. Introduction

There has been growing concern about the increased incidence of foodborne disease and the resistance of foodborne pathogens to drugs in the last decade. This has led to scientific and political efforts to address the problem, which may otherwise impact on public health and agriculture. One of the pathogens of concern belongs to the genus, *Arcobacter*; this genus was first described by Ellis in 1977 as a taxon that contains Gram-negative, spirillum-like bacteria isolated from bovine and porcine fetuses (Ellis *et al.*, 1977; Fera *et al.*, 2004). The first classification of *Arcobacter* was proposed by Vandamme *et al.* (1991) to describe those organisms previously classified as ‘aerotolerant campylobacters’. Although this genus has a role in foodborne diseases, the mechanisms they use to cause disease in animals and humans are still largely unknown; their mechanisms of antimicrobial resistance are also little known.

The significance of these organisms as a potential food safety problem is evidenced by the increasing number of reports on arcobacters published during last few years, which recognise arcobacters as emerging foodborne pathogens (Atabay *et al.*, 2006). In recent decades, *Arcobacter* species have become significantly more important in veterinary and human public health because they are potential zoonotic bacteria (Collado *et al.*, 2011; Houf *et al.*, 2005). In animals, arcobacters have been associated with abortion, gastrointestinal disorders and mastitis (Houf *et al.*, 2005; Vandamme *et al.*, 1992; Van Driessch *et al.*, 2003). Although *Arcobacter* species have been widely isolated from symptomatic cases such as diarrhoeic patients, aborted fetuses, and patients with bacteraemia (Schroeder *et al.*, 1996; Woo *et al.*, 2001; Lau *et al.*, 2002; Kopilovic *et al.*, 2008; Muzny *et al.*, 2010), infection caused by the genus is still considered to be underestimated because of the insufficiency in identification methods (Vandenberg *et al.*, 2004).

Since the complete genome of *Arcobacter butzleri* strain RM4018, isolated from a human clinical case was published by Miller and others (2007), particular emphasis has been focused on the physiology and genetics of this genus. *Arcobacter butzleri* is the most important species of the genus *Arcobacter* and has been categorized as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002).

1.1. Taxonomy of the genus *Arcobacter*

Taxonomy of the genus *Arcobacter* has taken on increasing importance as members of the genus have been recognised as emerging enteropathogens and potential zoonotic agents (Collado *et al.*, 2011; Ho *et al.*, 2006b; Snelling *et al.*, 2006). Formerly, arcobacters were classified as aerotolerant *Campylobacter* species (Neill *et al.*, 1985). There has been remarkable progress in understanding the taxonomy of this genus since it was first described by Vandamme *et al.* (1991). The genus *Arcobacter* belongs to the family *Campylobacteriaceae* together with the genera *Campylobacter* and *Sulfurospirillum* (Collado *et al.*, 2009a; Vandamme *et al.*, 2005). Species of *Arcobacter* have been isolated from a wide diversity of hosts and habitats such as the milk of cows with mastitis and the faeces, blood, reproductive tracts, and aborted fetuses of various farm animals (Higgins & Degre, 1979; Vandamme *et al.*, 1992; Wesley *et al.*, 1993; Skirrowii, 1994; Wesley, 1996; Collado *et al.*, 2011; Neill *et al.*, 1985). At the time of writing, the genus *Arcobacter* comprises seventeen recognised species (Table 1.1). Additionally, an obligate microaerophilic bacterium that oxidizes sulphides was proposed as a new species "Candidatus *Arcobacter sulfidicus*" in 2002 (Wirsén *et al.*, 2002), but the description does not officially exist yet.

The taxonomy of the genus *Arcobacter* has been based on the analysis of the 16S rRNA gene (Collado *et al.*, 2011; Wesley *et al.*, 1995). The seventeen recognised species of *Arcobacter* (Table 1.1) show high interspecies sequence similarities (92.0 to 98.8%) based on 16S rRNA gene sequences (Wesley *et al.*, 2005; Figure 1.1). Housekeeping genes such as those encoding the DNA-dependent RNA polymerase subunits β and β' (*rpoB-rpoC*) and DNA gyrase subunit A (*gyrA*), have also been used to bring a higher degree of resolution between the species (Morita *et al.*, 2004; Abdelbaqi *et al.*, 2007b). While these genes are highly conserved, they have higher levels of interspecies variation than is seen with the 16S rRNA genes and consequently have been used to provide better differentiation between the species, and to allow the phylogenetic relationships to be more clearly established. Phylogenetic analysis of the GyrA proteins of *Arcobacter butzleri* has shown them to be highly related to those of *Wolinella succinogenes* and *Helicobacter pullorum* (Abdelbaqi *et al.*, 2007b).

Table 1.1 Recognised species in the genus *Arcobacter*

Species	Type strain	Other designations	Source	References
<i>A. nitrofigilis</i>	LMG 7604	ATCC 33309; CCUG 15893; CECT 7204	Roots from <i>Spartina</i> <i>alterniflora</i> (Canada)	McClung <i>et al.</i> , 1983 Vandamme <i>et al.</i> , 1991
<i>A. cryaerophilus</i>	A169/B	D2792; ATCC 43158; LMG 24291; LMG 7536; LMG 9904	Brain, aborted bovine foetus (Ireland)	Neill <i>et al.</i> , 1985 Vandamme <i>et al.</i> , 1991
<i>A. butzleri</i>	D2686	RM4018; LMG 10828; ATCC 49616; CCUG 30485; CIP 103493	Faeces, human with diarrhoea (USA)	Kiehlbauch <i>et al.</i> , 1991 Vandamme <i>et al.</i> , 1992b
<i>A. skirrowii</i>	449/80	ATCC 51132; CCUG 10374; CIP 103538; LMG 6621	Faeces, lamb with diarrhoea (Belgium)	Vandamme <i>et al.</i> , 1992b
<i>A. cibarius</i>	LMG 21996	CECT 7203; CIP 108697; CCUG 48482	Broiler carcasses (Belgium)	Houf <i>et al.</i> , 2005
<i>A. halophilus</i>	ATCC BAA 1022	LA31B; CIP 108450; CCUG 53805	Hypersaline lagoon (USA)	Donachie <i>et al.</i> , 2005
<i>A. mytili</i>	CECT 7386	F2075; LMG 24559 CIP 110066	Mussels (Spain)	Collado <i>et al.</i> , 2009a
<i>A. thereius</i>	LMG 24486	CCUG 56902; 16398	Pig abortion (Denmark)	Houf <i>et al.</i> , 2009
<i>A. marinus</i>	JCM 15502	CL-S1; KCCM 90072	Seawater associated with starfish (Korea)	Kim <i>et al.</i> , 2010
<i>A. ellisii</i>	LMG 26155	CECT 7837	Mussels (Spain)	Figueras <i>et al.</i> , 2011a
<i>A. molluscorum</i>	LMG 25693	CECT 7696	Shellfish (Spain)	Figueras <i>et al.</i> , 2011b
<i>A. trophiarum</i>	LMG 25534	CCUG 59229	Fattening pigs (Belgium)	De Smet <i>et al.</i> , 2011b
<i>A. defluvii</i>	LMG 25694	CECT 7697	Sewage (Spain)	Collado <i>et al.</i> , 2011
<i>A. bivalviorum</i>	LMG 26154	CECT 7835	Mussels (Spain)	Levican <i>et al.</i> , 2012
<i>A. venerupis</i>	LMG 26156	CECT 7836	Clams (Spain)	Levican <i>et al.</i> , 2012
<i>A. cloacae</i>	SW28-13 ^T	LMG 26153, CEC ^T 7834 ^T	Sewage (Spain)	Arturo <i>et al.</i> , 2013
<i>A. suis</i>	F41 ^T	LMG 26152 ^T , CEC ^T 7833 ^T	Pork meat (Spain)	Arturo <i>et al.</i> , 2013

ATCC, American Type Culture Collection; CCUG, Culture Collection of the University Gothenburg, Gothenburg, Sweden; CECT, Spanish Type Culture Collection; LMG, Culture Collection of the Laboratory for Microbiology Gent, University of Gent, Gent, Belgium; CIP, Collection de l'Institut Pasteur.

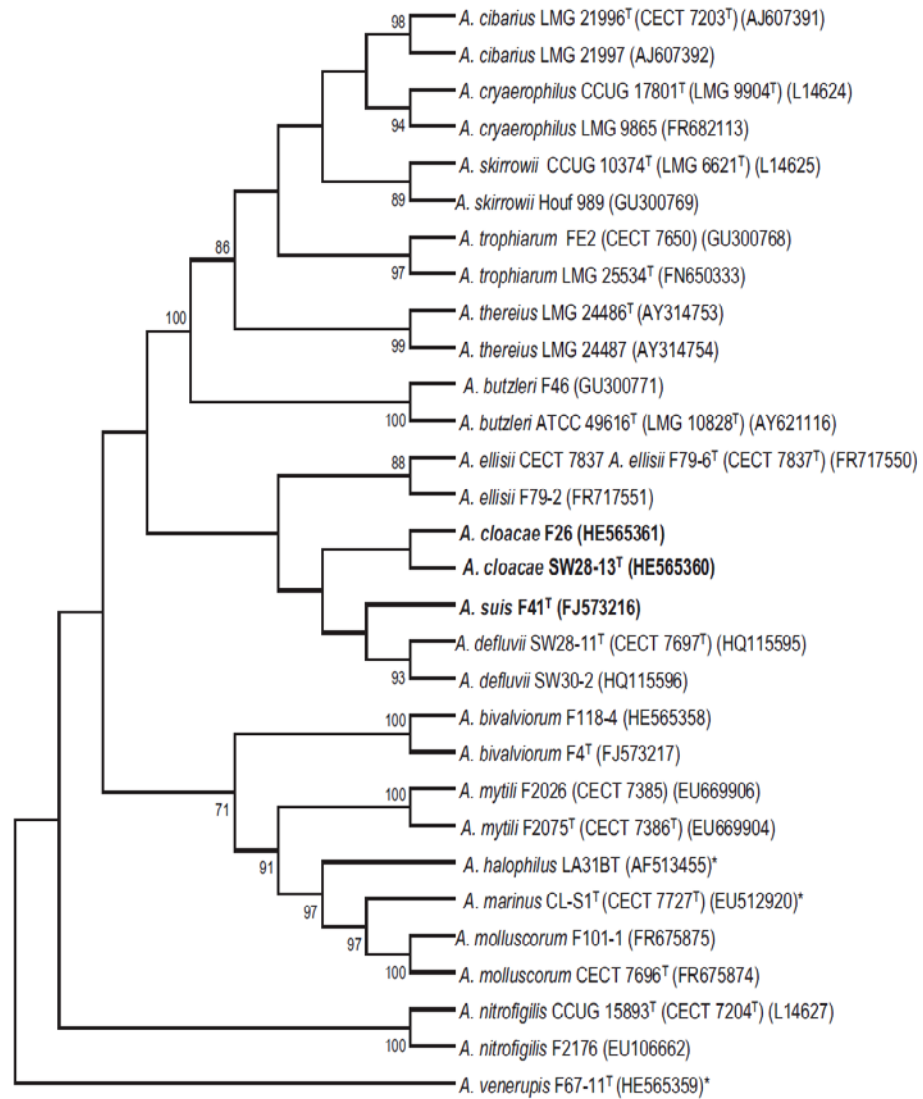


Figure 1.1 Neighbour-Joining tree based on 16S rDNA sequences of currently accepted *Arcobacter* species (from Arturo *et al.*, 2013).

1.2. Genomics

The analysis of genomic sequences is an important step in understanding the genetics and physiology of any organism that composes a link between the environments and hosts. *Arcobacter* species are widespread in many different environments such as marine water, fresh water and soil. As of 2013, four *Arcobacter* genomes have been completely sequenced; *A. butzleri* RM4018, *A. nitrofigilis* strain CI^T, *A. butzleri* ED-1 and *A. sp.* Strain L. (Toh *et al.*, 2011; Pati *et al.*, 2010; Miller *et al.*, 2007). The first (*A. butzleri* strain RM4018, GenBank_CP000361.1) was from a human clinical isolate and is a member of the epsilon subdivision of the *Proteobacteria*; it is a close taxonomic relative of recognized pathogens, such as *Campylobacter jejuni* and *Helicobacter pylori*. This strain belongs to the Family *Campylobacteraceae*, but the majority of its proteome is most closely related to two members of the *Helicobacteraceae*, *Sulfuromonas denitrificans* and *Wolinella succinogenes* (Miller *et al.*, 2007). The *Arcobacter butzleri* RM4018 genome comprises 2,341,251 bp and is considered to be the third largest genome within the *Epsilonproteobacteria*; it is predicted to encode 2,259 protein sequences (Pati *et al.*, 2010; Miller *et al.*, 2007; Table 1.2).

Genomic analyses have shown that a significant proportion of the *A. butzleri* genome is dedicated to growth and survival of the organism under diverse environmental conditions, with a large number of coding sequences encoding signal transduction, chemotaxis and respiration-associated proteins, along with proteins involved in DNA repair and adaptation. The *A. butzleri* RM4018 genome includes a number of genes involved in sulphur metabolism, which are more common within free-living taxa such as *Nitratiruptor*, *Sulfurovum* and also *Sulfurimonas* (Miller *et al.*, 2007). This suggests reassessment is needed to clarify whether the inclusion of *Arcobacter* in the family *Campylobacteraceae* is correct or not (Miller *et al.*, 2007; On *et al.*, 2009). This observation is also supported by the results of a study by Debruyne *et al.*, (2008) that identified *Sulfurimonas denitrificans* as the species most closely related to *A. butzleri* on the basis of a phylogenetic analysis of 60 genes from the *A. butzleri* RM4018 genome. Furthermore, putative virulence genes of *Arcobacter butzleri* strain RM4018 were recognized as homologous to those described for *Campylobacter* (Miller *et al.*, 2007), which suggests that *A. butzleri* can be correctly classified as an emerging pathogen.

The second *Arcobacter* genome to have been fully sequenced is that of *Arcobacter nitrofigilis* type strain CI^T (Pati *et al.*, 2010, GenBank_CP001999.1). This genome consists of 3,192,235 bp and is the second largest genome within the *Epsilonproteobacteria*. It consists of one circular chromosome with an overall G+C content of 28.4%. The genome of *A. nitrofigilis* is larger than those of both *A. butzleri* and *C. jejuni* (Miller & Parker, 2011). The *Arcobacter butzleri* ED-1 (GenBank_AP012047.1) genome consists of a circular 2,256,675-bp chromosome with a G+C content of 27.1% and contains 1,454 (67%) genes with function prediction out of 2,158 predicted protein-coding genes, 65 (3%) novel hypothetical genes and 639 (30%) conserved hypothetical genes (Toh *et al.*, 2011). The *Arcobacter* sp. strain L (GenBank_AP012048.1) genome consists of a circular 2,945,673-bp chromosome with a G+C content of 26.6%, containing 2,845 predicted protein-coding genes and a small plasmid (1,989-bp) containing three protein-coding genes. The *Arcobacter* sp. strain L genome contains 1,812 (64%) of functional predicted genes, 748 (26%) conserved hypothetical genes and 288 (10%) novel hypothetical genes and a region (2,051-bp) known as clustered regularly interspersed short palindromic repeats (Toh *et al.*, 2011).

Several genome sequencing projects are currently on-going for other *Arcobacter* isolates. In 2009, the *Campylobacter*, *Helicobacter* and Related Organism Congress (CHROC) reported the sequencing of a bovine strain of *A. butzleri* which showed considerable divergence from the human strain RM4018 (Sloane *et al.*, 2009). At the time of writing, the genomes of *A. butzleri* 7h1h and *A. butzleri* JV22 are also being sequenced (Merga *et al.*, from 2011, unpublished; Muzny *et al.*, from 2010, unpublished; NCBI).

Partial sequence data are available for *A. halophilus* LA31BT (Miller *et al.*, 2009) and comparative analysis suggests that there is a high degree of similarity between this strain and *A. butzleri* RM4018. Interestingly, although there are expected characteristics in common between the species, *A. halophilus* LA31BT showed multiple unique genes that require further investigation (Miller & Parker, 2011; Wesley & Miller, 2010). For example, preliminary analysis of the genomic sequence of *A. halophilus* LA31BT has revealed the presence of genes commonly associated with halotolerance and supports the observed halotolerance of this strain in contrast with *A. butzleri* RM4018. The arcobacters are aerotolerant species, in contrast to campylobacters and the sequence

information also indicates the presence of proteins predicted to be involved in aerotolerance in each of the *Arcobacter* genomes sequenced (Miller & Parker, 2011).

Table 1.2 Genomic analysis of *A. butzleri* (strain MR4018) *A. nitrofigilis* (strain CI^T), *A. butzleri* ED-1 and *A. sp.* strain L

General features	<i>A. butzleri</i> MR4018	<i>A. nitrofigilis</i> CI ^T	<i>A. butzleri</i> ED-1	<i>A. Sp. strain L.</i>
Genome size (bp)	2,341,251	3,192,235	2,256,675	2,945,673
DNA G+C content (bp)	27.05%	28.36%	27.1%	26.6%
Extrachromosomal elements	0	0	0	1
Protein-coding genes	2,259 (96.82%)	3,154 (97.83%)	2,158	2,845
Pseudogenes	5	70	68	74
Total genes	2,264	3,224	2,226	2,919
RNA genes	96 (4.11%)	70 (2.17%)	58 (2.60%)	61 (2.08%)
Conserved hypothetical genes	743 (33%)	-	639 (30%)	748 (26%)
Novel hypothetical genes	Many, uncounted	-	56 (3%)	288 (10%)
Genes with function prediction	1011 (43.33%)	2,324 (72.08%)	1,454 (67%)	1,812 (64%)

(Miller *et al.*, 2007; Pati *et al.*, 2010; Toh *et al.*, 2011)

1.3. Microbiological features of *Arcobacter* spp.

1.3.1. Epidemiology

Arcobacters have been isolated from many different sources such as animals and their products, human and clinical samples, fresh and marine waters, mussels, clams, and other foods (de Boer *et al.*, 1996; Kiehlbauch *et al.* 1991; On *et al.*, 1995; Atabay *et al.*, 1998; Yan *et al.*, 2000; Lau *et al.*, 2002; Oliveira *et al.*, 2003; Collado *et al.*, 2011). First isolation of *Arcobacter* was in Belfast, UK, from aborted bovine fetuses by Ellis *et al.* (1977) although it was not classified as an *Arcobacter* at the time, but rather as aerotolerant *Campylobacter* (Julia *et al.*, 1991). In many studies, arcobacters (*A. butzleri*, *A. cryaerophilus* and *A. skirrowii*) have been associated with animal diseases including abortion, mastitis and diarrhoea (Higgins & Degre, 1979; Vandamme *et al.*, 1992; Wesley *et al.*, 1993; Skirrowii, 1994; Wesley, 1996), and reproduction disorders (Oliveira *et al.*, 1997; Anonymous, 1998). *Arcobacter* species have been also isolated from vaginal swabs of healthy and normal reproducing sows (On *et al.*, 2002; Kabeya *et al.*, 2003). Besides the association with reproduction problems, one study has reported the presence of arcobacters in the stomachs of pigs with gastric ulcers (Suarez *et al.*, 1997); however, the evidence is ambiguous and needs to be confirmed. Arcobacters have been isolated from faeces of apparently healthy pigs of different ages (Hume *et al.*, 2001; Kabeya *et al.*, 2003; Van Driessche *et al.*, 2003; 2004; Ho *et al.*, 2006b) and from the mouth and the faeces of dogs, in which mostly *A. cryaerophilus* was found (Houf *et al.*, 2008).

Furthermore, several studies have demonstrated that arcobacters could be potentially zoonotic agents through their presence in food of animal origin, including poultry, turkey, pork, and beef (Atabay *et al.*, 1998; 2003; Manke *et al.*, 1999; Ohlendorf & Murano, 2002; Romero *et al.*, 2002; On *et al.*, 2003). Although knowledge of the clinical importance of *Arcobacter* spp. is still limited, arcobacters have been isolated from many clinical samples in both animals and humans. In addition, the International Commission on Microbiological Specifications for Food has indicated that *A. butzleri* is a 'serious hazard' to human health (ICMSF, 2002; On *et al.*, 2003); however, the significance of *Arcobacter* spp. as a cause of human disease is still unknown. For instance, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been isolated from the

faeces of humans with gastroenteritis and stomach cramps (Tee *et al.*, 1988; Kiehlbauch *et al.*, 1991; Vandamme *et al.*, 1992; Lerner *et al.*, 1994; Kiss and Csorian 1996; Lauwers *et al.*, 1996; Marinescu *et al.*, 1996; Mansfield *et al.*, 2000; Ian *et al.*, 2002; Wybo *et al.*, 2004; Fernandez *et al.*, 2004; Vandenberg *et al.*, 2004) and from human blood, including a case of neonatal bacteraemia (Kiehlbauch *et al.*, 1991; On *et al.*, 1995; Hsueh *et al.*, 1997). Moreover, while they have rarely been implicated in extra-intestinal invasive disease, Yan *et al.* (2000) reported *Arcobacter* spp. isolated from blood cultures of an elderly man with liver cirrhosis who suffered from fever and oesophageal variceal bleeding. Lau *et al.* (2002) also reported the isolation of *A. butzleri* from blood culture of a woman with acute gangrenous appendicitis (Lehner *et al.*, 2005).

In addition to the isolation of arcobacters from animals or food sources, many studies reported that *Arcobacter* species are associated strongly with fresh-water and marine environments (Jacob *et al.*, 1993; 1998; Rice *et al.*, 1999; Stampi *et al.*, 1999; Assanta *et al.*, 2002; Fera *et al.*, 2004; Mangeri *et al.*, 2000; 2005; Collado *et al.*, 2011). Water is a potential source of *Arcobacter* spp. (Anderson *et al.*, 1993; Rice *et al.*, 1999). *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. cibarius* have been isolated from various environmental sources, including surface water, groundwater, seawater, wastewater, and sewage treatment plants (Rice *et al.*, 1999; Mangeri *et al.*, 2000; Moreno *et al.*, 2003; Van Driessche *et al.*, 2003; Fera *et al.*, 2004; Houf *et al.*, 2005). Additionally, *Arcobacter butzleri* and *A. cryaerophilus* were isolated from a drinking water reservoir in Germany (Jacob *et al.*, 1998), canal water in Thailand (Dhamabutra *et al.*, 1992), river water in Italy (Musmanno *et al.*, 1997), ground water sources and sewage (Stampi *et al.*, 1993), and brackish lakes (Mangeri *et al.*, 2000). Furthermore, the recovery of arcobacters from drinking water reservoirs (Jacob *et al.*, 1993; 1998) has become a public health concern, especially as these organisms may attach to inner surfaces of pipes (Assanta *et al.*, 2002).

Many potentially novel *Arcobacter* species, based so far on only 16S rRNA sequence data (Miller *et al.*, 2007), have been identified in aquatic and extreme environments. These include; the flora of deep-sea hydrothermal vents (Moussard *et al.*, 2006), hydrocarbon-contaminated seawater (Prabakaran *et al.*, 2006), a low salinity petroleum reservoir (Grabowski *et al.*, 2005), infected or dead coral surfaces (Frias-Lopez *et al.*,

2002), deep-sea sediments (Thamdrup *et al.*, 2000), tube worms (Naganuma *et al.*, 1996), anaerobic sludge (Snaird *et al.*, 1997), and a circulated dairy wastewater lagoon (McGarvey *et al.*, 2005). Recently, new species of *Arcobacter* have been isolated from mussels, shellfish and clams (Collado *et al.*, 2009a; Figueras *et al.*, 2011a, 2011b; Levican *et al.*, 2012).

1.3.2. Morphology, isolation and phenotypic identification

The phenotypic characteristics of the genus *Arcobacter* show many of the characteristics of the family Campylobacteraceae (Vandamme and De Ley 1991; Phillips, 2001). *Arcobacter* spp. are generally Gram negative curved rods 1–3 by 0.2–0.9 μm in size (Figure 1.2) and display a corkscrew motility by having a single unsheathed polar flagellum (Phillips, 2001). After incubation at 30°C aerobically, the organism produces 2–4 mm grey or whitish colonies on blood agar (Vandamme *et al.*, 1993) and swarming may occur on fresh agar (Mansfield & Forsythe, 2000). The key distinguishing features of the genus *Arcobacter* used to differentiate them from *Campylobacter* are: the ability to grow at 15°C but not at 42°C and the ability to grow optimally aerobically at 30°C (Ursing *et al.*, 1994; Vandamme & De Lay, 1991; Vandamme *et al.*, 1991). *Arcobacter* species show morphological characteristics similar to *Campylobacter*, and also give the same positive reactions to the standard biochemical tests including oxidase, catalase, indoxyl acetate and Gram stain (Vandamme and De 1991).

Arcobacter culture is carried out by an enrichment procedure using a series of different enrichment media which can take from 2 to 5 days to isolate the organism (Lehner *et al.*, 2005). Several protocols have been developed to isolate organisms from this genus although there is currently no standard protocol for the selective isolation and quantification of arcobacters. For instance, cefsulodin-irgasan-novobiocin (CIN) agar, selective for *Yersinia* spp., has been used to recover arcobacters from pork meat samples (Collins *et al.*, 1996) and from faeces of humans with enteritis (Burnens *et al.*, 1992), but the original methods used for isolation of *Arcobacter* spp. are mainly based on those developed for *Campylobacter* species.

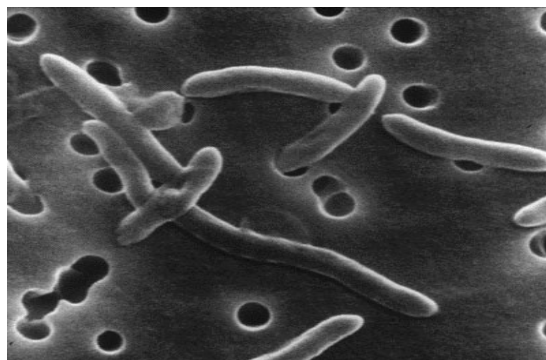


Figure 1.2 A scanning electron microscope image of *Arcobacter butzleri* (Marke & Dickinson, 1996).

Many enrichment media have been developed and supplemented with different ingredients and antibiotics such as cefoperazone, amphotericin, teicoplanin (CAT) or modified cefoperazone, charcoal, deoxycholate (mCCD) followed by plating onto solid media containing cefoperazone, trimethoprim, piperacillin and cycloheximide and incubation at 24°C (Steele & McDermott, 1984; Lammerding *et al.*, 1996; DeBoer *et al.*, 1996; Atabay & Corry, 1997; 1998). Frequently, *Arcobacter* broth (CM0965, Oxoid) with CAT or mCCD and EMJH medium (Appendix) with 5-fluorouracil have entirely supported growth of *Arcobacter* species although these supplements might not inhibit the associated flora in clinical and environmental samples (Kurt *et al.*, 2001).

Recently, Merga *et al.* (2011) have demonstrated that freezing a faecal sample will result in a 50% reduction in recovery of arcobacters. They have also compared five isolation methods which were named as follows: HH (Houf broth and Houf plates), HCC (Houf broth with mCCDA-CAT plates), ACH (*Arcobacter* broth- CAT broth with Houf plates), ACCC (*Arcobacter* broth-CAT broth with mCCDA-CAT plates) and CC (*Campylobacter*-specific broth and *Campylobacter*-specific plates), and shown that HCC was more selective for *A. skirrowii* than the HH method, which gave a much higher proportion of *Arcobacter butzleri*.

Generally, classical biochemical identification tests for campylobacters have been standardized (On and Holmes, 1991; On *et al.*, 1991) and tests for distinguishing *Arcobacter* species were proposed in *Bergey's Manual of Systematic Bacteriology*

(Vandamme *et al.*, 2005). The most useful biochemical tests for discriminating the currently recognized and lately proposed *Arcobacter* species are shown in Table 1.3 (Debruyne *et al.*, 2008; Arturo *et al.*, 2013; Levican *et al.*, 2012; Collado and Figuras, 2011).

Table 1.3 Phenotypic characteristics of all accepted *Arcobacter* species^a

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Growth conditions																	
O ₂ at 37°C	V	V	+	+	--	+	+	--	--	+	+	+	--	+	+	+	--
CO ₂ at 37°C	--	V	+	+	+	+	+	--	--	+	+	+	+	+	+	+	--
CO ₂ at 42°C	--	--	V	--	--	--	+	--	--	+	+	--	--	+	--	--	--
4%(w/v) NaCl	+	--	+	+	--	+	+	+	--	--	+	+	--	--	+	--	--
1%(w/v) glycine	--	--	--	--	--	+	+	+	V	--	--	--	--	--	+	--	--
MaConkey agar	--	V	+	--	+	+	+	+	V	+	+	--	+	V	--	+	+
Enzyme activity																	
Catalase	+	+	V	+	V	--	+ ^b	+	+	+ ^b	+	+	+	+	--	+	+
Urease	+	--	--	--	--	--	--	--	--	+	--	--	+	V	--	--	--
Nitrate reduction	+	+ ^b	+	+	--	+	+	+	--	+	+	--	+	+	+	+	+
Indoxyl acetate hydrolysis	+	+	+	+	+	+	--	+	+	+	--	+	+	+	+	+	+
Resistance to cefoperazone (64 mg/l)	--	+	+	+	+	--	--	+	+	V	+	--	--	--	--	--	--

1; *A. nitrofigilis*, 2; *A. cryaerophilus*, 3; *A. butzleri*, 4; *A. skirrowii*, 5; *A. cibarius*, 6; *A. halophilus*, 7; *A. mytili*, 8; *A. thereius*, 9; *A. trophiarum*, 10; *A. defluvii*, 11; *A. molluscorum*, 12; *A. bivalviorum*, 13; *A. venerupis*, 14; *A. ellisii*, 15; *A. marinus*, 16; *A. cloacae*, 17: *A. suis*.

+ ≥95% of strains positive

--≤11% of strains positive

V 12-94% of strains positive

^a Data from references (Arturo *et al.*, 2013; Levican *et al.*, 2012; Collado and Figuras, 2011).

^b weak reaction.

1.3.3. Molecular identification

Several molecular methods have been designed for *Arcobacter* identification at genus and species level. Identification of *Arcobacter* species has been established by using DNA-based assays such as one-step PCR, multiplex-PCR (Houf *et al.*, 2005), and amplified fragment length polymorphism (AFLP) although sequencing of 16S-23S rRNA (rDNA) molecules has led to accurate phylogenetic evaluation of the *Arcobacter* species (Wesley *et al.*, 1995; On *et al.*, 2004; Ongor *et al.*, 2004; Lehner *et al.*, 2005). The most widely used method is the one-step PCR (targeting the 16S and 23S rRNA genes), developed for the simultaneous detection and identification of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* by Houf *et al.* (2000). Despite this method being very popular it produces misidentification of *A. nitrofigilis* with *A. skirrowii* and also confuses the latter species with the recently proposed with *A. mytili* (Collado *et al.*, 2009a). Another multiplex-PCR for the identification of *Arcobacter* species considered of medical importance was described by Kabeya *et al.* (2003a). This method can differentiate between the two DNA groups of *A. cryaerophilus* but has not gained much popularity. Several other molecular methods for detection and identification of *Arcobacter* have recently been described. These include; PCR-DGGE (Petersen *et al.*, 2007), real time-PCR (Abdelbaqi *et al.*, 2007a, Brightwell *et al.*, 2007) and DNA microarray assay (Quiñonez *et al.*, 2007), although none of them allow the detection and/or identification of all the accepted *Arcobacter* species.

A recently proposed method based on the 16S rDNA-RFLP patterns differentiated all the accepted species, *A. butzleri*, *A. cryaerophilus*, *A. cibarius*, *A. skirrowii*, *A. nitrofigilis* and *A. halophilus* (Figueras *et al.*, 2008). This method has not only been successfully used for the identification of more than 600 *Arcobacter* strains in several studies (Figueras *et al.*, 2008; Collado *et al.*, 2008, 2009b, submitted) but has also enabled us to recognize new *Arcobacter* species such as *A. mytili* (Collado *et al.*, 2009a) and other new candidate species based on the new RFLP patterns observed. This method can also differentiate the new species *A. marinus*, which possesses a distinctive pattern after digestion of the 16S rDNA gene of this species with the *MseI* enzyme. However, this method cannot differentiate the recently described species *A. thereius* because it produces the same pattern as *A. butzleri* (Collado *et al.*, 2009b). These data demonstrate that until a new identification method is developed for the characterization

of the species with common RFLP patterns, the best way to identify the strains is to use both methods (m-PCR and the 16S rDNA-RFLP) in parallel and to further sequence the 16S rDNA or the *rpoB* genes in cases of incongruent results. So far, in our hands, this has been the only way to obtain accurate identification of all *Arcobacter* species.

1.3.4. Genotyping and genetic diversity

Various different methods have been used to distinguish one strain of *Arcobacter* from another, to allow researchers to study transmission routes, or for tracing sources of outbreaks. These include several PCR-based techniques, such as enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), randomly amplified polymorphic DNA-PCR (RAPD-PCR), AFLP, and pulsed-field gel electrophoresis (PFGE) (Houf *et al.*, 2002; On *et al.*, 2003, 2004; Hume *et al.*, 2001). Each of these methods has advantages and disadvantages related to its reproducibility, simplicity, discriminatory power, and cost (Houf *et al.*, 2002). The most commonly used typing technique has been ERIC-PCR, which has been successfully applied to the investigation of disease outbreaks (Vandamme *et al.*, 1993), the characterization of isolates from foods and water (Houf *et al.*, 2002; Aydin *et al.*, 2007), and to isolates included in the description of new *Arcobacter* species in order to find out whether they have a clonal origin (Houf *et al.*, 2005; 2009; Collado *et al.*, 2009a). However, in the recent description of the new species *A. trophiarum*, 10 different AFLP profiles were recognized among the 16 isolates recovered from this species, while with ERIC-PCR only 4 genotypes could be identified (De Smet *et al.*, 2010). These differences are surprising since ERIC-PCR has been the recommended technique for genotyping *Arcobacter* strains on the basis of results obtained in several studies (Houf *et al.*, 2010; Houf *et al.*, 2002). This indicates that the resolving power of these typing techniques should be re-evaluated for *Arcobacter* by sequencing these strains, since so far there are no comparative studies on this genus that provide evidence that AFLP has a better resolution than ERIC-PCR.

A website database for multilocus sequence typing (MLST) has recently been created by Miller *et al.* (Miller *et al.*, 2009; <http://pubmlst.org/arcobacter>), who analysed seven genes of 374 strains belonging to five species of the genus (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius*, and *A. thereius*). The website provides

information on primers and sequencing conditions for the seven genes (*aspA*, *atpA*, *glnA*, *gltA*, *pgm*, *tkt*, and *glyA*) and for submitting new sequences. The results of the MLST approach did not, however, find any association between the sequence types and the host or geographical sources, thus corroborating the high genetic diversity within the *Arcobacter* spp. reported in previous studies using other typing methods (Hume *et al.*, 2001; Houf *et al.*, 2002; On *et al.*, 2003; Aydin *et al.*, 2007). The wide variation in the genotypes may be due to multiple sources of contamination (Aydin *et al.*, 2007) or, as has been suggested for *Campylobacter*, to their ability to incorporate exogenous DNA or to undergo genomic rearrangement by multiple recombinations (Hume *et al.*, 2001). Further studies will be required to confirm the basis of this genetic variation in *Arcobacter* species (Collado *et al.*, 2010)

1.4. Veterinary and public health significance of *Arcobacter* infection

As has been described in the preceding sections, *Arcobacter* species are commonly isolated from food products of animal origin, which has led to classification of arcobacters as an emerging food pathogens. Three species, *A. butzleri*, *A. skirrowii* and *A. cryaerophilus*, have been associated with diseases of both humans and animals (Kiehlbauch *et al.*, 1991; Burnens *et al.*, 1992; Engberg *et al.*, 2000; Lehner *et al.*, 2005). Their presence in food or water can lead to a serious risk to public health with severe economic consequences in the veterinary field. However, up to now little is known about the mechanisms of pathogenicity or potential virulence factors of *Arcobacter* species. Epidemiologically, *A. butzleri* is the most important species of the genus and has been categorized as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002).

1.4.1. *Arcobacter* species in humans

Arcobacter species have been isolated from symptomatic and asymptomatic people in many different countries (Vandamme *et al.*, 1992; On *et al.*, 1995; Wybo *et al.*, 2004; Samie *et al.*, 2007). The prevalence of *Arcobacter* in human infections has probably been underestimated previously because of inappropriate detection and typing methods

applied to stool samples (Vandamme *et al.*, 1993; Phillips 2001; Vandenberg *et al.*, 2004). Three species, *A. butzleri* and, more rarely *A. skirrowii* and *A. cryaerophilus*, have all been associated with human enteritis and occasionally bacteraemia (Lerner *et al.*, 1994; On *et al.*, 1995; Wybo *et al.*, 2004; Lehner *et al.*, 2005).

A. butzleri shows microbiological and clinical features similar to those of *Campylobacter jejuni*; however, *A. butzleri* is more frequently associated with persistent and watery diarrhoea (Olivier *et al.*, 2004) and less often with more serious bloody diarrhoea (Vandenberg *et al.*, 2004). Patients with *A. butzleri* infection have reported suffering from diarrhoea associated with abdominal pain, with the occurrence of a variety of symptoms including nausea, vomiting and fever, sometimes sufficiently severe to require hospitalization (Vandenberg *et al.*, 2004; Vandamme *et al.*, 1992; Collado, 2010). Olivier *et al.* (2004) identified *A. butzleri* as one of the most common organisms isolated from human stool specimens while other studies have demonstrated that *A. butzleri* is the fourth most common *Campylobacter*-like organism recovered from human stools of patients with diarrhoea (Vandenberg *et al.*, 2004; Prouzet-Mauleon *et al.*, 2006). *A. cryaerophilus* has also been found in stool specimens of patients with diarrhoea as well as from the blood of a neonate and a reported case of bacteraemia (On *et al.*, 1995; Lau *et al.*, 2002). *A. skirrowii* has been isolated from an elderly patient with chronic diarrhoea (Wybo *et al.*, 2004) and from patients with HIV in South Africa (Samie *et al.*, 2007) although in this case the source of the *A. skirrowii* remains ambiguous.

However, *Arcobacter* species have also been isolated from faeces of healthy people (Vandenberg *et al.*, 2004; Samie *et al.*, 2007). For instance, *A. cryaerophilus* was isolated from asymptomatic individuals working in a slaughterhouse in Switzerland (Houf and Stephan, 2007) and in a study on Belgians, human asymptomatic carriage of *A. butzleri* was shown to be more frequent than that of *C. jejuni* (Vandenberg *et al.*, 2004). Relatively little is known on the global contribution *Arcobacter* species have towards human health (Vandenberg *et al.*, 2004). As is the case with other pathogens, host factors such as immune status and age might play a role in determining whether *Arcobacter* infection can take place, although this has not yet been proven (Vandenberg *et al.*, 2004; Kownhar *et al.*, 2007).

Despite the fact that isolation procedures specific for the recovery of arcobacters are seldom applied in routine clinical laboratory testing, there is increasing evidence to suggest a substantive role for *Arcobacter* in human illness (Vandamme *et al.*, 1993; Phillips 2001). Because of the small number of studies that have been performed, it is impossible to establish whether or not prevalence varies between developed and developing countries. Currently, *Arcobacter* species are not considered a major health concern; this is because of lack of adequate detection and identification methods needed to isolate *Arcobacter* from clinical specimens. To enhance recovery and proper identification of *Arcobacter* species, a standardised isolation method must be established to determine the true role of these species in human disease.

1.4.2. *Arcobacter* species in animals

In the last few years, *Arcobacter* species have increasingly frequently been isolated from different livestock animals and pets, even given the lack of strong detection and identification methods. *Arcobacter* species have been isolated from healthy animals and also associated with clinical conditions including abortion, mastitis, diarrhoea and reproduction illnesses (Higgins & Degre, 1979; Vandamme *et al.*, 1992; Wesley *et al.*, 1993; Skirrowii, 1994; Wesley, 1996; Oliveira *et al.*, 1997; Anonymous, 1998). *Arcobacter* species have been isolated from various different animals and their discharges such as faeces, saliva and vaginal discharge (On *et al.*, 2002; Kabeya *et al.*, 2003; Ho *et al.*, 2006a). Arcobacters have been isolated from faeces of apparently healthy pigs in different ages (Hume *et al.*, 2001; Kabeya *et al.*, 2003; Van Driessche *et al.*, 2003; 2004; Ho *et al.*, 2006b) and from the mouth and the faeces of dogs, in which mostly *A. cryaerophilus* was found (Houf *et al.*, 2008). Besides the association with reproduction problems, one study has reported the presence of arcobacters in the stomachs of pigs with gastric ulcers (Suarez *et al.*, 1997); however, the evidence is ambiguous and needs to be confirmed.

Occasionally, *A. butzleri* has been associated with enteritis and diarrhoea in pigs, cattle, and horses while *A. skirrowii* has been associated with diarrhoea and haemorrhagic colitis in sheep and cattle (Vandamme *et al.*, 1992b; Ho *et al.*, 2006a). *Arcobacter butzleri* is the only species isolated both from healthy non-human primates and from

those with diarrhoea in several studies (Anderson *et al.*, 1993; Higgins *et al.*, 1999; Wesley *et al.*, 2003; Stirling *et al.*, 2008). The recently described species *A. thereius* was also recovered from liver and kidney of spontaneous porcine abortions, but despite no other established abortifacient agents being detected, the pathogenic role of this recently described species also remains unknown (Houf *et al.*, 2009). Additionally, *Arcobacter trophiarum* has been also isolated recently from faeces of fattening pigs (De Smet *et al.*, 2011b). It has been cited in previous section that *Arcobacter* could cause clinical symptoms that include abortion, mastitis and diarrhoea (Logan *et al.*, 1982; Vandamme *et al.*, 1992; Collado, 2010). *Arcobacter* species have consistently been isolated from the intestinal tract and faecal samples of different farm animals, but apparently only have the capacity to cause disease in some of them. Certain *Arcobacter* species have been isolated from different animals; *A. cryaerophilus* has frequently been associated with animals while *A. butzleri* and *A. skirrowii* are less common (Oliveira *et al.*, 1997; On *et al.*, 2003).

Faecal shedding of *Arcobacter* is well known in poultry such as chickens, ducks, turkeys, and domestic geese (Festy *et al.*, 1993; Atabay *et al.*, 2006, 2008). However, there have been no reports of a link between faecal shedding and disease in these birds, which may indicate that poultry is a natural reservoir of *Arcobacter* species (Atabay *et al.*, 2006, 2008). Similarly, although *Arcobacter* has been isolated several times from cases of bovine abortion (Ellis *et al.*, 1977; Neill *et al.*, 1985; Fernandez *et al.*, 1995b), the bacteria have also been recovered from healthy bovine foreskin washings (Gill 1983) as well as from vaginal swabs of cows with no observable reproductive problems (Kabeya *et al.*, 2003b).

1.4.3. Transmission routes of *Arcobacter* infection

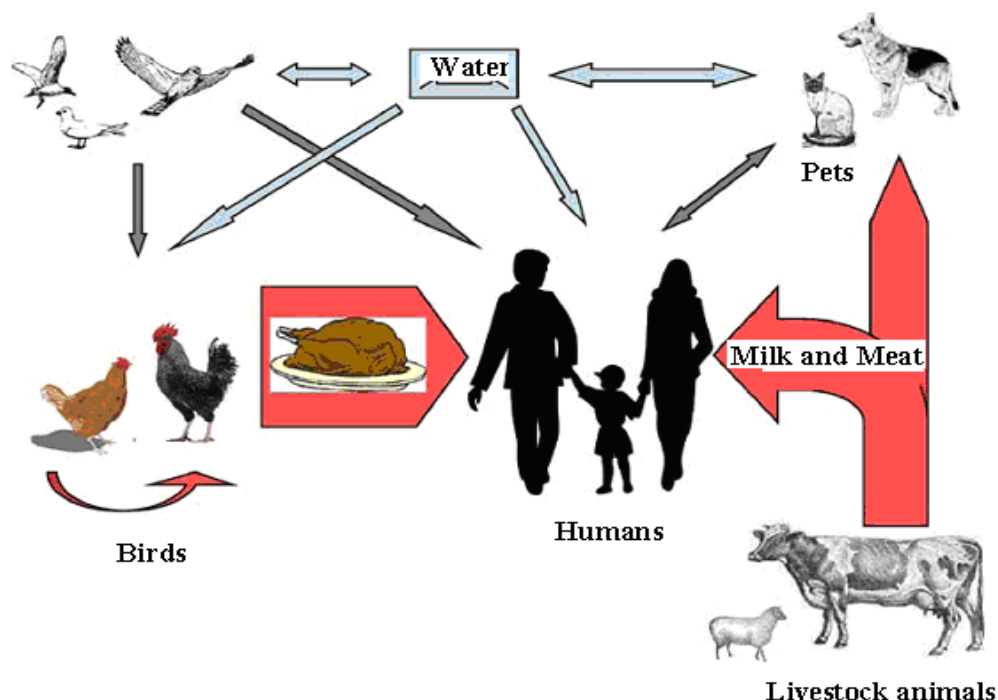


Figure 1.3 Proposed routes of transmission of *Arcobacter* spp.

1.4.3.1. Food

As we have seen (Figure 1.3), food products of animal origin are considered an important potential transmission route of *Arcobacter* species (Ho *et al.*, 2006a; Lehner *et al.*, 2005), which are common in the intestinal tract and faecal samples of healthy as well as diseased animals (Lehner *et al.*, 2005; Van Driessche *et al.*, 2003; Hume *et al.*, 2001). Several studies have demonstrated that contamination of meat products by *Arcobacter* species most likely occurs when faecal material comes into contact with carcasses during slaughter (Van Driessche *et al.*, 2007; Lehner *et al.*, 2005; Ohlendorf and Murano, 2002). Worldwide, there is increasing evidence that livestock animals are significant reservoirs of *Arcobacter* species and over the last few years the presence of these organisms in food products of animal origin including raw meat products (beef, pork and chicken meat) has received increasing attention (Duffy and Fegan, 2012; Morita *et al.*, 2004; Lehner *et al.*, 2005; Snelling *et al.*, 2006). The rate of isolation from

chicken meat has been shown to be significantly higher than from beef and pork (Kabeya *et al.*, 2004). Atanassova *et al.* (2008) examined five broiler flocks and five turkey flocks in the course of slaughtering and processing, and found that 43.0% of the broilers and 18.2% of the turkeys sampled were positive for *A. butzleri*, *A. skirrowii* and *A. cryaerophilus*. *A. cibarius* has also been isolated from broiler carcasses (Houf *et al.*, 2005). In certain studies, whilst the only *Arcobacter* species isolated from minced beef samples was *A. butzleri*, all three pathogenic species, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*, were detected in rectal swabs (Ongor *et al.*, 2004). Moreover, *Arcobacter* species have been isolated from raw cows' milk with *A. butzleri* as the dominant species followed by *A. cryaerophilus* (Scullion *et al.*, 2006; Shah *et al.*, 2012a).

Several investigations have also shown *A. butzleri* and *A. cryaerophilus* are present on slaughter equipment (Houf *et al.*, 2003) and cross contamination can occur from contact of raw ingredients with the processing equipment. Recently, shellfish have also been identified as a potential source of *Arcobacter* infection especially as they are traditionally eaten lightly cooked or raw (Levican *et al.*, 2012; Figueras *et al.*, 2011; Shah *et al.*, 2011; Iwamoto *et al.*, 2010; Collado *et al.*, 2009a; Fernandez *et al.*, 2001). The prevalence of *Arcobacter* in other foods is largely unknown, mainly because *Arcobacter* is not routinely tested for in foods. Interestingly, Gonzalez and Ferrus (2011) report having detected *Arcobacter* species in fresh vegetables such as lettuces (20% of the samples they tested were positive for *Arcobacter* spp.). These foods are generally considered safe and, indeed, *Arcobacter* contamination levels seem to be rather lower in these vegetable samples than in animal-derived food products or water samples. Any of these food or water sources could be considered as having a potential public health risk associated with them and further studies are needed to take into consideration the risks of *Arcobacter* contamination in these foods (Gonzalez and Ferrus, 2011).

Table 1.4 Prevalence of *Arcobacter* isolation from different animal origin food sources

Country	Type of food samples	<i>Arcobacter</i> positive (%)	References
Australia	Beef	22	Rivas <i>et al.</i> , 2004
	Lamb meat	15	Rivas <i>et al.</i> , 2004
	Pork meat	29	Rivas <i>et al.</i> , 2004
	Chicken meat	73	Rivas <i>et al.</i> , 2004
Belgium	Beef	31.3	Collado <i>et al.</i> , 2009b
	Minced beef	9	De Smet <i>et al.</i> , 2010
	Rabbit meat	10	Collado <i>et al.</i> , 2009b
	Pork meat	53	Collado <i>et al.</i> , 2009b
	Dairy cattle milk	3.18	Pianta <i>et al.</i> , 2007
Egypt	Rabbit meat	75	Iman Suelam, 2012
Japan	Beef	2.2	Kabeya <i>et al.</i> , 2004
	Chicken meat	49	Morita <i>et al.</i> , 2004
	Pork meat	7	Kabeya <i>et al.</i> , 2004
Korea	Chicken meat	22.2	Lee <i>et al.</i> , 2010
Malaysia	Beef	38	Shah <i>et al.</i> , 2010
	Beef	26.3	Shah <i>et al.</i> , 2012a
	Dairy cattle milk	7.6	Shah <i>et al.</i> , 2012a
Mexico	Beef meat	28.8	Villarruel-Lopez <i>et al.</i> , 2003
	Pork meat	51.5	Villarruel-Lopez <i>et al.</i> , 2003
	Chicken meat	40	Villarruel-Lopez <i>et al.</i> , 2003
Northern Ireland	Beef	34	Scullion <i>et al.</i> , 2006
	Dairy cattle milk	46	Scullion <i>et al.</i> , 2006
	Pork meat	35	Scullion <i>et al.</i> , 2006
	Chicken meat	62	Scullion <i>et al.</i> , 2006
Netherlands	Minced beef	1.3	De Boer <i>et al.</i> , 1996
Spain	Beef	31.3	Collado <i>et al.</i> , 2009b
	Pork meat	53	Collado <i>et al.</i> , 2009b
	Chicken meat	64	Gonzalez <i>et al.</i> , 2000
	Duck meat	40	Collado <i>et al.</i> , 2009b
	Turkey meat	33.3	Collado <i>et al.</i> , 2009b
	Clam	100	Collado <i>et al.</i> , 2009a
	Mussels	41.1	Collado <i>et al.</i> , 2009a
Turkey	Minced beef	37	Aydin <i>et al.</i> , 2007
	Chicken meat	68	Aydin <i>et al.</i> , 2007
	Turkey meat	4	Aydin <i>et al.</i> , 2007
Thailand	Chicken meat	100	Mrita <i>et al.</i> , 2004
USA	Pork meat	30	Ohlendorf and Murano, 2002
	Ground pork	54	Collins <i>et al.</i> , 1996
	Chicken meat	84	Johnson and Murano, 1999

1.4.3.2. Water

Water can play an important role in the transmission of pathogenic bacteria and contaminated drinking water has been cited as a major contributing factor for diseases caused by a number of bacterial species including *Arcobacter* (Lehner *et al.*, 2005). Many studies have shown the presence of *Arcobacter* in various sources of water such as surface water, ground water, drinking water, raw sewage and sea water (Shah *et al.*, 2012b; Collado *et al.*, 2010; Lenher *et al.*, 2005; Morita *et al.*, 2004; Fera *et al.*, 2004; Stampi *et al.*, 1993; Rice *et al.*, 1999; Jacob *et al.*, 1998; Musmanno *et al.*, 1997). Furthermore, several studies have found that water acts as a vehicle for transmitting *Arcobacter* to both humans and animals (Shah *et al.*, 2012b; Figure 1.3). *Arcobacter* species have been detected in countries across the globe. In Japan and Thailand, *A. butzleri* was found in 23% (4/17) of samples of Japanese river water tested and in 100% (7/7) of Thai canal waters sampled (Morita *et al.*, 2004). *A. butzleri* has also been isolated in South Africa from drinking water reservoirs, in water treatment plants, rivers and well water (Diergaardt *et al.*, 2004). In Germany, *Arcobacter* strains isolated from drinking water treatment plants were shown to have the same serotypes as those observed from human isolates (Jacob *et al.*, 1998).

It is thought that *Arcobacter* was the cause of at least three water-borne disease outbreaks around the world (Kopilovic *et al.*, 2008; Fong *et al.*, 2007; Rice *et al.*, 1999). Rice and others were the first to report the outbreak at a Girl Scout Camp in Idaho (USA); affected individuals presented with symptoms of gastroenteritis (nausea, vomiting, abdominal cramps and diarrhoea). In this case, the automated chlorination system for water at the camp had broken down and *A. butzleri* was isolated from the well (ground-water) that was used as the source for drinking water, suggesting a causal link for the outbreak (Rice *et al.*, 1999). Another outbreak, this time in Ohio (USA) was thought to be of multiple aetiologies (Fong *et al.*, 2007), where *Arcobacter* species were isolated from samples taken from the well heavily polluted by faecal material. In Slovenia, *A. cryaerophilus* as well as numerous other pathogens were found in stool samples of patients affected in an outbreak thought to be caused by the contamination of drinking water that was connected to the building they lived in (Kopilovic *et al.*, 2008). A recent study conducted by Collado and Figueras (2011), found that the incidence of *A. butzleri* and *A. cryaerophilus* was high in Llobregat River water (a main source of

drinking water for the metropolitan area of Barcelona, Spain). Van Driessche and Houf (2008) have demonstrated that *Arcobacter*'s capacity to survive in water is influenced by multiple factors, including the presence of organic matter and temperature. Thus, under optimal conditions, arcobacters can be viable for up to 250 days. All the previous disease outbreaks of *Arcobacter* species associated with water are thought to be the result of faecal contamination. Due to faecal contamination, *Arcobacter* species can easily be introduced into different water sources, providing a route to *Arcobacter* infection of both animals and humans (Donachie *et al.*, 2005; Snelling *et al.*, 2006). It has been demonstrated that *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* were significantly more common in water sources where there was evidence of faecal contamination (Collado *et al.*, 2008; 2009a).

It has been suggested that drinking water treatment effectively removes these microorganisms from the finished chlorinated water because arcobacters are highly susceptible to chlorine (Rice *et al.*, 1999; Moreno *et al.*, 2004; Andersen *et al.*, 2007; Collado *et al.*, 2010), although *Arcobacter* has been isolated from treated drinking water after carbon filtration (Jacob *et al.*, 1998; Diergaardt *et al.*, 2004; Aydin *et al.*, 2007;). Despite that, treatment processes used to produce drinking water were adequate for removing *Arcobacter* species from the finished water (Collado *et al.*, 2010); it is still unknown whether or not conventional procedures for treating drinking water can effectively remove this bacterium as commented by Ho *et al.* (2006a).

1.4.3.3. Contact

In addition to consumption of undercooked or raw food products of animal origin and drinking water, contact with persons and animals is considered as another potential source of *Arcobacter* infection (De Smet *et al.*, 2011a; Fera *et al.*, 2009; Houf *et al.*, 2008). Person to person transmission has been suggested after an Italian outbreak at a children's nursery associated with recurrent gastroenteritis related symptoms such as abdominal cramps. The strains recovered from the faecal matters of those infected patients showed the same phenotype and genotype (Vandamme *et al.*, 1993; 1992a). Another study has also suggested person to person transmission of *A. butzleri* in a neonate thought to have been transmitted via placenta (On *et al.*, 1995). Furthermore,

contact with pets, through faeces or saliva, is thought to be a potential route for *Arcobacter* transmission (Figure 1.3). A small number of studies found a high prevalence of *Arcobacter* species in faeces and oral swabs of pets (cats and dogs); this led to the proposal that *Arcobacter* might be transmitted through contact with these pets (Fera *et al.*, 2009; Fernández *et al.*, 2008). However, this is opposite to previous studies which showed no *Arcobacter* growth in both saliva and faeces samples from cats (Aydin *et al.*, 2007; Houf *et al.*, 2008). Additionally, wild animals could contribute in the epidemiology features of *Arcobacter* species (Collado and Figueras, 2011; Hamir *et al.*, 2004). Another study has demonstrated that *Arcobacter* was also reported in exotic, non-domesticated animals e.g. Galapagos turtles, rhinoceros, gazelle and alpaca (Wesley *et al.*, 2003).

1.5. Antimicrobial resistance

Antimicrobials have provided a significant improvement in public health over the last 50 years. In this success also lies a problem since their overuse and misuse have led to resistance in bacterial populations (Levy, 1990; 1986). Antibiotic resistant variants of common bacteria are more populous now than they were even a decade ago and certainly more than when antibiotics were first introduced as therapy (Cohen, 1992; Neu, 1992). However, studies on antimicrobial resistance of *Arcobacter* species are lacking although some preliminary studies have been done on antimicrobial sensitivity of *Arcobacter* species against certain antibiotics. For instance, *A. butzleri* RM4018 has shown resistance against 42 of 65 antibiotics, a level that is remarkably high compared to some *Campylobacter* species (William *et al.*, 2007; Fouts *et al.*, 2005). In another study by Insook (2007), arcobacters isolated from broiler carcasses were 94% resistant to at least one or more antibiotics and 72% were resistant to two or more antibiotics. Furthermore, Fera *et al.* (2003) tested the antimicrobial sensitivity of 30 isolates of *Arcobacter* species from brackish environments against 26 different antimicrobial agents and all strains showed high resistance to chloramphenicol, penicillin, trimethoprim, macrolide, and vancomycin. Atabay and Aydin (2001) also found that 100% of *A. butzleri* isolates from chickens were resistant to sodium cefuroxime, trimethoprim/ sulphamethoxazol, aztreonam and penicillin G, while 26% of *A. butzleri* isolates were resistant to ampicillin, amoxicillin, clavulanic acid and amoxicillin.

Furthermore, for *A. butzleri* RM4018, antibiotic resistance is a consequence of the presence of genes known to play a role in antibiotic resistance. For instance, genes encoding three putative β -lactamases (AB1486, AB1306 and AB0578) have been identified in the RM4018 genome and are likely to result in β -lactam resistance; the presence of the *lrgAB* genes, which encode antiholin-like proteins have also been shown to cause β -lactam resistance in other bacterial species (Balyes, 2000; Groicher *et al.*, 2000). Chloramphenicol resistance results from the presence of a *cat* gene encoding a chloramphenicol O-acetyltransferase, and also the presence of the *upp* gene which encodes uracil phosphoribosyl-transferase (Glaab *et al.*, 2005; Martinussen & Hammer, 1994).

Additionally, organic acids (>0.2% acetic and citric acid) have been shown to be useful tools for reducing *A. butzleri* contamination (Cervenka *et al.*, 2004). In a study, six human isolates of pathogenic *Arcobacter* species (four strains of *A. butzleri* and two strains of *A. cryaerophilus*) were exposed to pH levels of 3.5-8.0 (D'Sa and Harrison, 2005) and most strains grew between pH 5.5 and 8.0. It has also been observed that there is a decrease in thermotolerance at pH 5.5, with D-values of 0.03– 0.11 min at 60°C, 0.30–0.42 min at 55°C and 1.97– 4.42 min at 50°C (Snelling *et al.*, 2006). Moreover, mild heat (50°C) followed by cold shock (4 or 8°C exposure) had a synergistic lethal effect, killing more cells than an individual 50°C treatment or with cold shock temperatures of 12 or 16°C (Snelling *et al.*, 2006).

In contrast, several antibiotic susceptibility tests have shown that *Arcobacter* species are sensitive to aminoglycosides, including kanamycin and streptomycin, which is similar to what has been reported for *Campylobacter* (Thwaites and Frost 1999; Kabeya *et al.*, 2004). The presence of acquired resistance to erythromycin and ciprofloxacin among poultry isolates is a matter of concern, for the reason that these antimicrobials are commonly suggested as first-line drugs for the treatment of *Campylobacter* infections in humans (Houf *et al.*, 2004). In addition, cephalothin should be used in the enrichment medium especially for *A. cryaerophilus* and *A. skirrowii* which were shown to be considerably more susceptible to this antibiotic than *A. butzleri* (Kabeya *et al.*, 2004).

1.6. Pathogenicity of *Arcobacter* spp.

Bacterial pathogenicity depends on many important requirements including the attachment to mucous surfaces, entry into host tissue through these surfaces, multiplication and dissemination *in vivo*, interference with host defence mechanisms, and damage to the host (Zhang, 2001; Kim *et al.*, 2000). As has been described in Section 1.3.1, the three species of the genus considered to be pathogenic (*Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii*) cause a range of conditions including enteritis, bacteraemia and reproductive disorders in animals, but the mechanisms they use to infect, multiply and disseminate within the host, are still poorly understood.

Since *Arcobacter* species are closely related to *Campylobacter* and *Helicobacter* species, and are included in the RNA Superfamily VI of the Proteobacteria (Phillips, 2001), *Arcobacter* may have virulence determinants in common with these species. The use of experimental animal models or cell lines may prove to be as valuable for investigating the pathogenesis of arcobacters as they have in helping evaluate the pathogenicity of *Campylobacter* and *Helicobacter* spp. (Babakhani *et al.*, 1993; Eaton *et al.*, 1991; 1992).

1.6.1. Putative virulence determinants

The mechanism of virulence in arcobacters is still a matter of argument. Several studies have investigated the virulence determinants associated with organisms of the *Campylobacter* genus, but there is a lack of knowledge about potential virulence factors in the genus *Arcobacter*. It has been suggested that if *Arcobacter*-related illnesses have a similar clinical pathology and outcome to those caused by *Campylobacter*, that there may be some conservation between the virulence factors of *C. jejuni* and those found in arcobacters (William *et al.*, 2007; Forsythe, 2006; Lehner *et al.*, 2005). Indeed, whole genome studies of *A. butzleri* strain RM4018 have identified a series of virulence determinants homologous with those of *C. jejuni* within this strain (William *et al.*, 2007). These include the fibronectin binding proteins and Cj1349.

Scrutiny of the *A. butzleri* RM4018 genome sequence suggests the presence of homologs of the *C. jejuni* invasion antigen protein (CiaB), inner-membrane protein (MviN), phospholipase PldA and haemolysin TlyA within this strain (William *et al.*, 2007). However, it has not yet been demonstrated whether these putative virulence determinants are functional and have a role in pathogenicity of this strain, regardless of the similarity to their campylobacter homologs. Other additional putative virulence determinants including: iron-regulated outer membrane virulence protein (IrgA), adhesion/haemagglutinin-HecA family and haemolysin activation protein (HecAB) have also been identified within in the *A. butzleri* RM4018 genome. The iron-regulated outer membrane protein encoded by *irgA* in *Vibrio cholerae* and the siderophore esterase (IroE) protein in *E. coli* are both candidates to play a role in the pathogenesis of urinary tract infections (Goldberg *et al.*, 1990; Johnson *et al.*, 2005; Larsen *et al.*, 2006). HecA is a member of the filamentous haemagglutinin family which widely contribute to both plant and animal pathogens in adherence, aggregation and epidermal cell killing and *hecB* encodes a related haemolysin activation protein (Rojas *et al.*, 2002).

Although some virulence factors identified in *C. jejuni* have homologs within the genome of *Arcobacter butzleri* strain RM4018, several campylobacter virulence-associated genes were not identified including the genes encoding the cytolethal distending toxin CDT-ABC (Miller *et al.*, 2007). This agreed with Johnson and Murano (2002) who were not able to detect *cdt* genes in some *Arcobacter* species using PCR and also this strain contains no cell-binding factor1 (CBF1) or JlpA (polypeptide jejuni lipoprotein) adhesin homologs (Jin S. *et al.*, 2001; Pei Z. *et al.*, 1993). Therefore, much research is still needed to better understand the mechanism of pathogenesis in *Arcobacter* species.

Other studies have investigated the putative virulence factors of particular *Arcobacter* isolates. For example, Musmanno *et al.* (1997) examined river water isolates of *A. butzleri* for putative virulence factors. Only one strain induced cytotoxic effects in tissue culture and one isolate caused elongation and adhered to cell lines. In another study by Johnson and Murano (2002), isolates from many different clinical and environmental samples were tested and toxicity to INT-407 and Hela cells was detected although cytolethal distending toxin (CDT) genes were not identified. The same study also

suggested that *Arcobacter* spp. might produce a protein that could be toxic to some cells lines; however the mechanism of action was different to that observed for *Campylobacter* CDT (Lehner, 2005). Villarruel (2003) examined the cytotoxicity of *Arcobacter* species isolated from meat; 95% of the isolates induced effects on Vero cells, including cell elongation, while a smaller proportion of the same isolates produced both vacuolization and elongation. Moreover, Ho *et al.* (2008) have found that *A. butzleri* possess two genes (*flaA* and *flaB*) coding flagellins like *Campylobacter* and *Helicobacter* species which were involved in their motility. Carbone *et al.* (2003), studied the ability of *A. butzleri* and other aquatic isolates, to adhere to and induce cytotoxicity in cultured epithelial cells. They reported that six of 27 of the *A. butzleri* strains could adhere to cell lines (Hela and Hep2 cells) and five of them produced toxic factors that changed the morphology of Vero cells, but all isolates were from aquatic environments and the number of strains was too low to make firm conclusions. The haemagglutinin protein has also been described in arcobacters as an immunogenic protein (Tsang *et al.*, 1996; Phillips, 2001).

1.6.2. Fibronectin-binding proteins

The adhesion of bacteria to epithelial cells is a most important step in human and animal colonization. Although the attached state promotes nutrient uptake and multiplication (Zobell, 1943), pathogens also use adherence as a direct tissue attack mechanism and to promote the delivery of toxins (Svanborg *et al.*, 1999; Linder *et al.*, 1988; Svanborg-Eden *et al.*, 1976; Smith & Linggood, 1972). Many pathogenic microorganisms have the ability to bind to the host extracellular matrix, and during the initial phases of infection this interaction occurs through fibronectin (Fn), collagen, vitronectin, and laminin.

1.6.2.1. Fibronectin

Fibronectin (Fn) is a glycoprotein present in a soluble form in plasma or as insoluble filaments in the extracellular matrices of vertebrates (Yasunori *et al.*, 2005). It is a high-molecular weight glycoprotein (220-440 kDa) and is considered to be a potential binding site for enteropathogenic bacteria because of its presence between the cells in the gastrointestinal epithelial layer (Quaroni *et al.*, 1978; Konkel *et al.*, 1997). Several studies have found that the molecules that comprise the fibronectin family play critical roles in cellular processes such as proliferation, migration, differentiation and adhesion (Debra, 1997; Hynes, 1990). Fibronectin exists as a dimer, consisting of two structurally similar, but non-identical folded polypeptide chains, each approximately 250 kDa in size. Each chain ends in a pair of C-terminal disulphide bonds and has about 30 intrachain disulphide bonds as well as two sulfhydryl groups (Debra, 1990). Structurally, each folded chain consists of groups of amino acids that form multiple repeating motifs or “modules” of three types (types I, II and III), that are grouped into functional domains (Figure 1.4). Modules of type I and type II are composed of two anti-parallel β -sheets stabilized by two intra-chain disulphide bonds, while type III also comprises two anti-parallel β -sheets, but this time lacking the disulphide bonds (Yong and Schwarzbauer, 2005; Baro *et al.*, 1990; Potts *et al.*, 1999).

Several studies have demonstrated that Fn matrix assembly is a complex process involving binding domains and repeating modules from all regions of Fn which participate in interactions with cell surface receptors such as transmembrane integrin receptors (Yong and Schwarzbauer, 2005). For instance, integrin $\alpha 5 \beta 1$ is the primary receptor for mediating assembly on the cell surface (Fogerty *et al.*, 1990; McDonald *et al.*, 1987), by attaching to one of the fibronectin-binding domains located in III₁₀ (Figure 1.5). Therefore, the interaction between Fn and $\alpha 5 \beta 1$ integrin plays a significant role in cell differentiation, migration and proliferation (Pierini *et al.*, 2000; Molla and Block, 2000; Garcia *et al.*, 1999) by serving as a connection between the cell and the extracellular matrix that mediates bidirectional signalling events through inside-out and outside-in pathways (Feiya *et al.*, 2003; Fernandez *et al.*, 1998). Furthermore, fibronectin type III modules bind to $\alpha 1 \beta$ integrins (membrane-spanning receptor proteins) on the cell membrane, while fibronectin-binding proteins usually attach to the N-terminal region of fibronectin (Danny *et al.*, 1999).

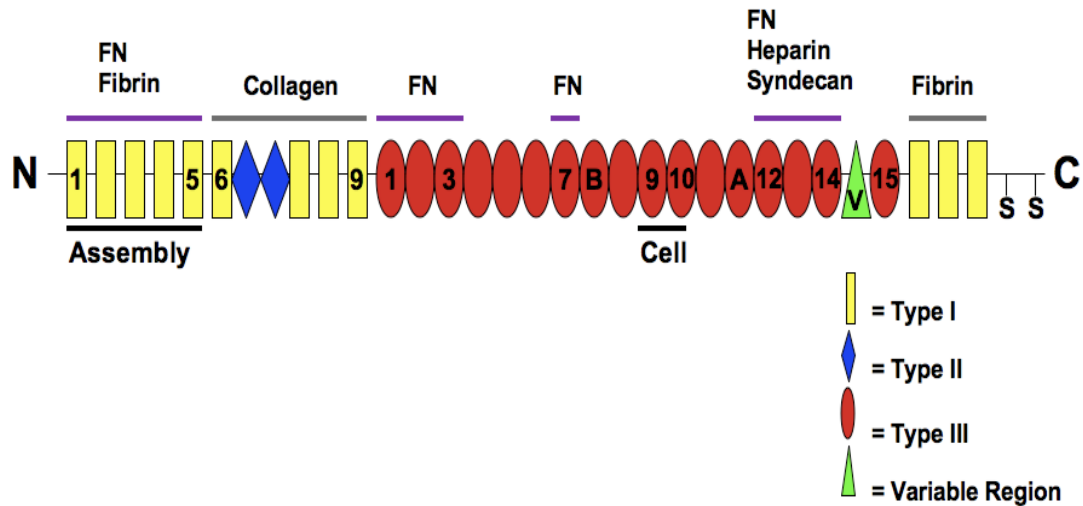


Figure 1.4 The modular structure of Fibronectin and its binding domains (Yong and Schwarzbauer, 2005), showing regions of the molecule form domains that interact with other molecules such as fibrin, collagen, cells and heparin; dimer forms via cysteine pair at the C-terminus (SS). Fibronectin molecule consists of 12 type I modules, 2 type II modules and 15–17 type III modules. The numbers represent the number of a series of types I, II, III repeats of amino acids characterize the structure of fibronectin molecule.

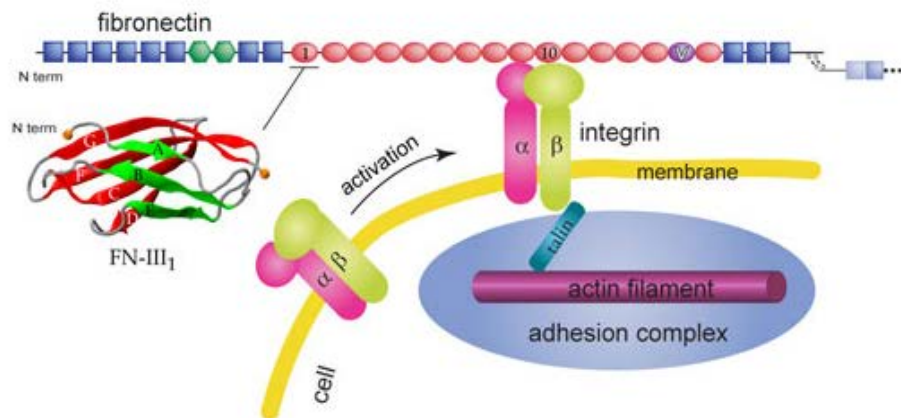


Figure 1.5 The interaction between Fibronectin modules and integrin on cell surface (Theoretical and Computational Biophysics Group <http://www.ks.uiuc.edu/Research/fibronectin/>). Sites of interaction include the assembly domain in I₁₋₅ as well as III₁₋₂ and III₁₂₋₁₄. The cell-binding domain includes III₁₀ and III₉ with the integrin binding sites (5α1β). A small diagram of folded proteins represents the ribbon structure of human FN type III₁₀ module.

1.6.2.2. Fibronectin-binding proteins (FnBP) in bacteria

Bacterial adhesins represent significant virulence factors in several pathogens (Zhang, 2001) and promote the release of toxins and lead to penetration of host cells (Carbonea *et al.*, 2003). Therefore, the adherence of pathogenic bacteria to host tissues is considered as a critical first step in their pathogenicity (Danny *et al.*, 1999). Adhesion to the intestinal epithelium is an important determinant of virulence for many pathogenic bacteria, allowing them to resist the fluid flow of luminal contents (Kim *et al.*, 2000). Most bacterial adhesins recognize and bind to host extracellular matrix (ECM) and other cell surface molecules such as fibronectin (Fn), collagen, fibrinogen/fibrin, elastin, laminin, vitronectin and heparin sulphate-containing proteoglycans (Joh *et al.*, 1999). These bacterial adhesins which bind to ECM molecules are known as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs); however, most of these molecules have not been identified and characterised (Danny *et al.*, 1999; Patti *et al.*, 1994; Patti and Hook, 1994). One of the extracellular matrix molecules that binds to MSCRAMMs, is fibronectin (Fn) which has been detailed in Section 1.6.2.1.

Several pathogenic bacteria have the ability to adhere to fibronectin (Fn) including *Salmonella enteritidis*, *Mycobacterium avium*, *Escherichia coli*, *Streptococcus pyogenes*, *Clostridium difficile*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Campylobacter jejuni*, and *Treponema* species (Baloda *et al.*, 1985; Schorey *et al.*, 1996; Visai *et al.*, 1991; Jaffe *et al.*, 1996; Hennequin *et al.*, 2003; Rydén *et al.*, 1983; van Putten *et al.*, 1998; Konkel *et al.*, 1997; Dawson & Ellen, 1994). The first discovery of bacterial FnBP was *Staphylococcus aureus* (Kuusela *et al.*, 1978) and the next genus shown to bind fibronectin was the *Streptococci* (Talay *et al.*, 1992). So far, over 100 bacterial FnBP have been identified (Brian *et al.*, 2011), and more than 10 FnBP have been just recognized in streptococci (Schwarz-Linek *et al.*, 2006). *Staphylococcus aureus* has two recognized genes which were named *fnbA* and *fnbB* (Nashev *et al.*, 2004; Peacock *et al.*, 2000; Jansson *et al.*, 1991; Kuusela *et al.*, 1978) and both code for large proteins (>1000 residues). Many other bacteria including Gram-positive (Table 1.5) and Gram-negative (Table 1.6) bacteria have one or more genes encoding FnBPs that bind fibronectin molecules in order to bypass the epithelial barriers and colonize the mammalian hosts.

Table 1.5 Gram-positive fibronectin-binding proteins

Bacterium	Fibronectin-binding protein	Mass (kDa)	References
<i>A. pyogenes</i>	CbpA	127	Esmay <i>et al.</i> , 2003; Pietrocola <i>et al.</i> , 2007
<i>Bac. fragilis</i>	Omp	102	Brain <i>et al.</i> , 2011
<i>Cl. difficile</i>	Fbp68(FbpA)	68	Hennequin <i>et al.</i> , 2003
<i>Cl. perfringens</i>	FbpA, FbpB	25, 66	Katayama <i>et al.</i> , 2009
<i>Ent. faecium</i>	SagA	53	Zareba <i>et al.</i> , 1997; Styriak <i>et al.</i> , 2004
<i>Er. rhusiopathiae</i>	RpsA, RpsB	219, 85	Shimoji <i>et al.</i> , 2003
<i>Lb. brevis</i>	SlpA	46	Engelhard, 2007
<i>Lb. plantarum</i>	enolase	-	Esgleas <i>et al.</i> , 2008
<i>L. monocytogenes</i>	FbpA, Five undefined proteins	55,-	Gilot <i>et al.</i> , 1999,2000; Dramsi <i>et al.</i> , 2004
<i>Mycobacteria spp.</i>	Antgen85ABCD, MPT51,Apa/FAP, antigen, Malate synthase	Wag22 31, 30, 31, 28, 30, 71, 80	Wiker and Harboe, 1992; Ohara <i>et al.</i> , 1995; Schorey <i>et al.</i> , 1995, 1996;; Belisle <i>et al.</i> , 1997; Rosseels <i>et al.</i> , 2006; Peake <i>et al.</i> , 1993; naito <i>et al.</i> , 2000; Ronning <i>et al.</i> , 2000; Anderson <i>et al.</i> , 2001; Kinhikar <i>et al.</i> , 2006
<i>Mycoplasma spp.</i>	EfTu, PDH- β , Hlp3, PlpA	30, 45, 200, 160	Razin <i>et al.</i> , 1998; Dallo <i>et al.</i> , 2002; May <i>et al.</i> , 2006; Balasubramanian <i>et al.</i> , 2008
<i>P. acnes</i>	undefined protein	80	Yu <i>et al.</i> , 1997
<i>S. aureus</i>	FnBPAB, Eap, Emp, Ebh, Aaa/Sle1	106, 104, 15, 36, 1100, 36,	Kuusela <i>et al.</i> ,1979; Espersen and Clemmensen, 19982; Flock <i>et al.</i> , 1987; Jonsson <i>et al.</i> , 1991; Peacock <i>et al.</i> , 2000; Nashev <i>et al.</i> , 2004

Table 1.5 (Continued) Gram-positive fibronectin-binding proteins

Bacterium	Fibronectin-binding protein	Mass (kDa)	References
<i>S. caprae</i>	AtlC	154	Allignet <i>et al.</i> , 2001
<i>S. epidermidis</i>	Aaa/Aae, Embp/Ebh	35, 1000	Heilmann <i>et al.</i> , 2003; Christner <i>et al.</i> , 2010
<i>S. saprophyticus</i>	Aas(AtlC), UaFA	159, 230	Aas Hell <i>et al.</i> , 1998; Gatermann and Meyer, 1994; Hell <i>et al.</i> , 1998
<i>Strep. agalactiae</i>	ScpB	22	Brown <i>et al.</i> , 2005
<i>Strep. dysgalactiae</i>	FnBA, FnBB	120, 125	Baums <i>et al.</i> , 2006; Lindgren <i>et al.</i> , 1992,1993
<i>Strep. equi ssp. equi</i>	FnE, FnEB, SFS	-, 53, 40	Lindmark <i>et al.</i> , 2001; Lannergard <i>et al.</i> , 2005; Lindmark and Guss, 1999
<i>Strep. equi ssp. zooepidemicus</i>	FNZ, FNZ2	65, 54	Natanson <i>et al.</i> , 1995; Lindmark <i>et al.</i> , 1996; Hong, 2005
<i>Strep. gordonii</i>	CshA, CshB, FbpA	265, 244, 63	McNab <i>et al.</i> , 1994; McNab and Jenkinson, 1992
<i>Strep. intermedius</i>	Antigen I/II	143	
<i>Strep. mutans</i>	Antigen I/II, FbpA/SmFnB, FBP-130	166, 63, 130	Miller-Torbert <i>et al.</i> , 2008
<i>Strep. pneumonia</i>	PavA(FbpA), SP-0082, PbfA	63, 91, 74	Lau <i>et al.</i> , 2001; Bumbaca <i>et al.</i> , 2004; Yamaguchi <i>et al.</i> , 2008
<i>Strep. pyogenes</i>	PrtF1/SfB1, PrtF2, FbaA, FbaB/PrtF2-Like, Serum opacity factor, Sfbx , Fbp54 (FbpA), GAPDH, Protein H, M1 protein, Shr, 28-kDa protein	69,128,38,81,112,72, 54,36,37,55,143,28	Switalski <i>et al.</i> , 1982; Talay <i>et al.</i> , 1991,1992; Hanski <i>et al.</i> , 1992; Hanski and Caparon, 1992; Katerov <i>et al.</i> , 1998; Sela <i>et al.</i> , 1993; Ozeri <i>et al.</i> , 1996; Talay <i>et al.</i> , 2000; Dinkla <i>et al.</i> , 2003; Schwarz-Linek <i>et al.</i> , 2006
<i>Strep. suis</i>	FbpS(FbpA), OFS, Enolase	64, 117, 48	Staats <i>et al.</i> , 1997; De Greef <i>et al.</i> , 2002; Sun <i>et al.</i> , 2005

Table 1.6 Gram-negative fibronectin-binding proteins

Bacterium	Fibronectin-binding protein	Mass (kDa)	Reference
<i>A. butzleri</i>	Putative CadF, Cj27	37, 47	William <i>et al.</i> , 2007
<i>B. henselae</i>	BadA, Pap31	328, 29	Batterman <i>et al.</i> , 1995; Dabo <i>et al.</i> , 2006
<i>B. burgdorferi</i>	BBK32, RevA/B	42, 17	Szczepanski <i>et al.</i> , 1990; Coburn <i>et al.</i> , 2005; Fischer <i>et al.</i> , 2006
<i>C. jejuni</i>	CadF,Cj1279c	34, 46	Konkel <i>et al.</i> , 1997,1999a
<i>E. coli</i>	Curli, P-fimbriae, Pili(HCP), H6/H7 pili, Tsh, Upag	-, -, -, 142, 178	Froman <i>et al.</i> , 1884; Olsen <i>et al.</i> , 1989; Visai <i>et al.</i> , 1991; Westerlund <i>et al.</i> , 1991; Sokurenko <i>et al.</i> , 1992,1994; Otto <i>et al.</i> , 2005; Valle <i>et al.</i> , 2008
<i>H. ducreyi</i>	DrsA	26	Bauer and Spinola, 1999; Cole <i>et al.</i> , 2002; Leduc <i>et al.</i> , 2008,2009
<i>H. influenzae</i>	Pili, Hap	-, 154	Virkola <i>et al.</i> , 2000; St Geme, 2002
<i>Hel. pylori</i>	VacA toxin	136	Papini <i>et al.</i> , 2001
<i>L. interrogans</i>	LigA, LigB, LenB/C/E, LipL32	78, 201, 25, 32	Faine <i>et al.</i> , 1999; Merien <i>et al.</i> , 2000; Palaniappan <i>et al.</i> , 2002; Matsunaga <i>et al.</i> , 2003; Stevenson <i>et al.</i> , 2007; Tung <i>et al.</i> , 2010
<i>N. gonorrhoeae</i>	OpaA	27	Van Putten <i>et al.</i> , 1998
<i>Pasteurellaceae spp.</i>	ComE1	12	Redfield <i>et al.</i> , 2005; Mullen <i>et al.</i> , 2006,2007

Table 1.6 (Continued) Gram-negative fibronectin-binding proteins

Bacterium	Fibronectin-binding protein	Mass (kDa)	Reference
<i>P. multocida</i>	Omp87,Omp16	87, 16	Dabo <i>et al.</i> , 2005;
<i>Por. gingivalis</i>	Fimbrillin/FimA	37	Sojar <i>et al.</i> , 1995; Wu and Fives-Taylor, 2001
<i>Prev. intermedia</i>	AdpB	29	Yu <i>et al.</i> , 2006; Hirayama <i>et al.</i> , 2009
<i>Pesud. aeruginosa</i>	OprD, OprE1, OprE2, OprF	37-44	Roger <i>et al.</i> , 1999; Rebiere-Huet <i>et al.</i> , 1999, 2002, 2004
<i>Sal. typhimurium</i>	ShdA, MisL	207, 101	Kingsley <i>et al.</i> , 2000,2002; Boyen <i>et al.</i> , 2006
<i>Tann. forsythia</i>	BspA	114	Sharma <i>et al.</i> , 1998
<i>Trep. denticola</i>	OppA, Msp	67, 58	Umemoto <i>et al.</i> , 1993; Fenno <i>et al.</i> , 1998, 2000; Edwards <i>et al.</i> , 2005
<i>Trep. pallidum</i>	Several unidentified	32-90	Peterson <i>et al.</i> , 19983, 1987; Thomas <i>et al.</i> , 1985ab; Cameron <i>et al.</i> , 2004; Brinkman <i>et al.</i> , 2008
<i>V. vulnificus</i>	OmpU	35	Sperandio <i>et al.</i> , 1995; Goo <i>et al.</i> , 2006
<i>Y. pseudotuberculosis</i>	YadA	41	Tertti <i>et al.</i> , 1992

1.6.2.3. *Campylobacter* adhesion to fibronectin (CadF)

Several studies showed that *C. jejuni* produces a variety of adhesins such as CadF, FlpA, JipA and PEB1 (Konkel *et al.*, 1997; Pei *et al.*, 1998; Poly and Guerry, 2008). CadF protein is the most well characterized *C. jejuni* factor interacting with host cells (Scott *et al.*, 2010; Tadhg and Steffen, 2012). *Campylobacter* adhesion to fibronectin (CadF) was first identified in 1997 (Konkel *et al.*, 1997), is a 37 kDa outer-membrane protein and consists of two domains: an N-terminal transmembrane domain that forms a β -barrel pore in other proteins and a C-terminal domain forming a mixed α/β fold which then allows these bacteria to colonize and infect the intestinal mucosa (Nadeau *et al.*, 2003; Konkel *et al.*, 2004, 2005; Hu *et al.*, 2005). Studying the sequence of the *cadF* gene revealed a protein with a homology to an adhesin from *Pseudomonas fluorescens* (Tadhg and Steffen, 2012). Some studies have indicated that these pathogens can bind to fibronectin (Fn) of T84 human colonic cells (Konkel *et al.*, 2005). A year later, this observation was confirmed by another group, stating that recombinant CadF binds to fibronectin, but also can bind to a membrane fractions of INT-407 cells *in vitro* (Moser *et al.*, 1997).

Furthermore, another study revealed the importance of CadF for colonization; this was evident as *cadF* mutants were unable to infect chickens (Ziprin *et al.*, 1999). It was also reported that by using the polarized cell line T84, *C. jejuni* crossed the epithelial layer between neighbouring cells (paracellular route) causing invasion of the cells through the basolateral surface but not the apical surface (Monteville and Konkel, 2002). The previous study also has shown that CadF expression was required for maximal T84 cell binding. Moreover, Konkel *et al.* (2005) have also demonstrated that CadF contains a particular fibronectin-binding domain of 4 amino acids (FRLS) which possess Fn-binding activity. Mutations in these residues significantly reduce binding to fibronectin and INT-407 cells while peptides containing this fibronectin binding site exhibit a capacity to block binding. Interestingly, it was reported by Krause-Gruszczynska *et al.* (2007) that a 39-bp insertion in the *cadF* gene of *C. coli* strains leads to the formation of a protein that made these strains bind and invade INT-407 cells less efficiently than *C. jejuni*. In *C. jejuni*, *cadF* expression has been reported to be down-regulated in response to human mucin Muc2, suggesting that levels of protein expression may vary during an infection cycle (Tu *et al.*, 2008). Furthermore, most strains of *C. jejuni* exhibit two

bands in Western blots, using CadF antibodies, suggesting that distinct forms of the protein may indeed exist. A recent study has also revealed that CadF may undergo post-translational processing steps which in certain cases, results in the removal of the immunogenic epitope but retention of the fibronectin binding site (Scott *et al.*, 2010). Monteville *et al* (2003) suggested that a focal adhesion signalling molecule, paxillin, exhibited increased levels of tyrosine phosphorylation upon *C. jejuni* infection, which was not seen during infection with mutants of the fibronectin binding protein CadF; these data also indicate that there is a major signalling cascade (CadF-Fibronectin-Integrin β 1-FAK (Focal Adhesion Kinase)-Paxillin pathway) involved in events controlling *C. jejuni* internalization and host cell entry (Figure 1.6).

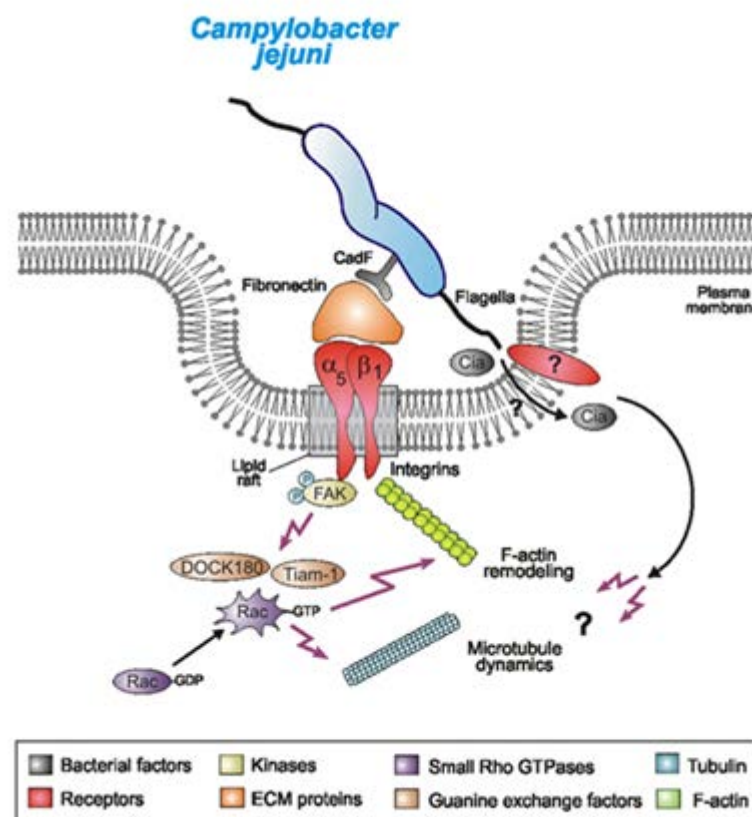


Figure 1.6 Proposed model for CadF induced signalling leading to bacterial invasion by *Campylobacter jejuni* (from Manja *et al.*, 2011). Several indicated host cell signalling molecules and pathways including the intracellular survival in *Campylobacter*-containing vacuoles (CCVs) have been reported in *in vitro* infection models and may play a role during pathogenesis *in vivo*. These data collectively suggest that the CadF→fibronectin→integrin-β1→FAK→paxillin pathway is another major signalling cascade involved in *C. jejuni*-mediated host cell entry.

1.6.2.4. Adhesion of *Arcobacter* spp. and CadF in their pathogenicity

A few studies have found that some *Arcobacter* species have ability to adhere to epithelial cells *in vitro* such as Hep-2 and Hela line cells (Carbone *et al.*, 2003; Houf and Stephan, 2006) although further investigation is needed to assess the significance of the *in vitro* adhesion capacity of *Arcobacter* species in their pathogenicity. For instance, Carbone *et al.* (2003) have noted that only six out of 17 of marine environment isolates of *A. butzleri* were able to adhere to both cells (Hep-2 and Hela line cells) although it has not yet been possible to differentiate whether the effect is caused by extracellular products or by cell-surface components. A later study investigated the potential of *A. cryaerophilus* strains to adhere to Hep-2 and Caco-2 cells, and four strains out of seven showed adhesion to at least one line cell (Houf and Stephan, 2006). Furthermore, it is thought that most previous outbreaks of *Arcobacter* may have resulted from faecal contamination, in addition to their ability to adhere to pipes and form biofilms (Assant *et al.*, 2002; Cervenka *et al.*, 2008; Shah *et al.*, 2011).

A putative *cadF* gene (AB0483) has been identified in *A. butzleri* RM4018 (Figure 1.7) and has been revealed to have significant homology with *Campylobacter jejuni* (57% identity) and *Wolinella succinogenes* (59% identity) genes encoding outer membrane proteins which adhere to fibronectin of intestinal epithelial cells (Monteville *et al.*, 2003; Konkel *et al.*, 2005; Miller *et al.*, 2007). Recently, a *cadF* gene has been identified in many different strains of *Arcobacter* from humans and animals such as cattle, sheep, chickens, horses and dogs (Doudah *et al.*, 2012); this study showed that *cadF* was identified in isolates of *A. butzleri* (100%), *A. cryaerophilus* (34%), and *Arcobacter skirrowii* (21%). The same study showed no significant differences for the *cadF* gene of *A. butzleri* strains isolated from both humans and animals. In addition, the *cadF* gene was also detected in *Arcobacter cryaerophilus* and *Arcobacter skirrowii* from cattle and dogs (Doudah *et al.*, 2012).

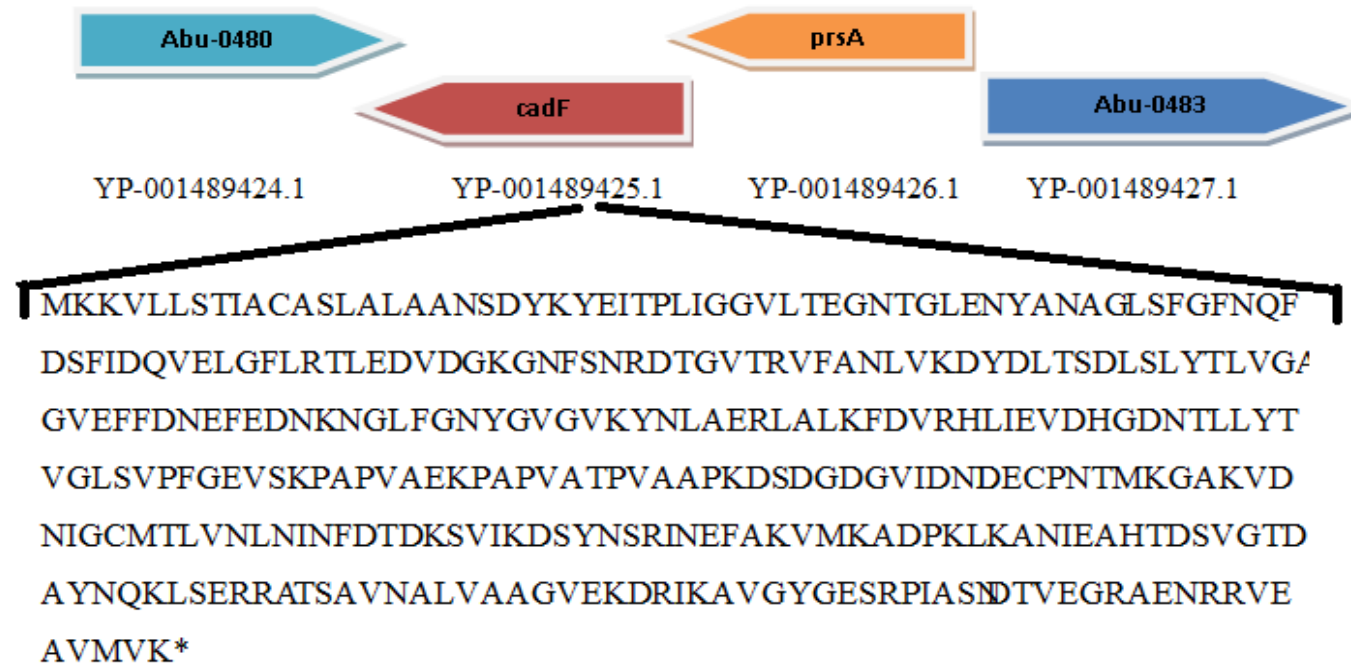


Figure 1.7 Position of *cadF* gene within genomic DNA of type strain *A. butzleri* RM4018 and outer membrane fibronectin-binding protein (CadF) sequence (NCBI).

1.7. Aims and objectives of the project

1.7.1. Aims

The overall aim of this project is to isolate and characterise environmental isolates of *Arcobacter* from Scottish surface waters and to investigate their pathogenicity profiles compared with those of the three type strains (*A. butzleri* strain D2686, *A. cryaerophilus* strain A169/B and *A. skirrowii* strain 449/80), which are known to cause disease in human and animal hosts. This project will investigate the pathogenicity determinants of the type strains and water isolates by examining the strains for the production of extracellular proteins known to be associated with virulence in closely related enterobacterial pathogens including *Campylobacter jejuni*. The study will particularly focus on the *cadF* gene which will be cloned, sequenced and mutagenised in the type strain *A. butzleri* and in environmental isolates that are recovered from the waters, to assess the role of the CadF protein in adherence of *Arcobacter* strains to epithelial cells of the host. This information will ultimately be relevant for the development of effective antimicrobial strategies to control this bacterium.

1.7.2. Specific Objectives

1. Isolation, distribution and heterogeneity of *Arcobacter* from environmental sources (surface water e.g. lakes, water reservoirs).
2. Characterisation of type strains and environmental isolates for the presence of putative virulence determinants.
3. Characterisation of the *Arcobacter* homolog of the *Campylobacter* fibronectin-binding protein CadF.

CHAPTER 2 MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strains

Bacterial strains in this study were a combination of laboratory strains, environmental isolates, and genetically modified *Arcobacter* and *Escherichia coli*. The strains from all studies are outlined in table 2.1.

Table 2.1 Bacterial strains used in this study

Bacterial Strains	Description	Source
<i>A. butzleri</i> D2686	(NCTC 12481), from faeces, human with diarrhoea.	National Collection of Type Cultures (NCTC)
<i>A. cryaerophilus</i> A169/B	(NCTC 11885), from bovine aborted foetus.	NCTC
<i>A. skirrowii</i> 449/80	(NCTC 12713), from faeces, lambs with diarrhoea.	NCTC
<i>Bacillus subtilis</i> DSM10	(NCTC 3610), original strain reference: Marburg	School of Life Sciences teaching lab strain collection
<i>E. coli</i> EMG 14	(ATCC 23737) Genotype: <i>K-12 met-λ-λ-r F</i>	School of Life Sciences teaching lab strain collection
<i>E. coli</i> XL1-Blue MRF	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' Δ proAB lacI ^q ZAM15 Tn10 (Tet ^r)]	Stratagene
<i>E. coli</i> BL21 (DE3)	<i>E. coli B F dcm ompT hsdS(r_B. m_B.) gal</i> Δ (DE3)	Novagen
SW-DL2	<i>Arcobacter spp.</i> isolate from Scottish surface water (Duddingston Loch)	This study
SW-OL2	<i>Arcobacter spp.</i> isolate from Scottish surface water (Loch Ore)	This study
D2686- Δ <i>cadF</i>	Wide type strain D2686 <i>cadF</i>	This study
SW_OL2- Δ <i>cadF</i>	Water isolate SW_OL2 <i>cadF</i>	This study

2.1.2. Plasmids

The plasmids used in this study are divided into two groups, cloning and mutagenesis vectors provided from commercial and academic sources (Table 2.2) and recombinant plasmids constructed in this study (Table 2.3). The maps of the plasmids are shown in Figures 2.1, 2.2, and 2.3.

Table 2.2 Plasmids used in this study

Cloning vectors	Description	Source
pBluescript KS(+)	Amp ^R , <i>lacZ</i> , T7 and T3 promoter, pUC origin of replication.	Stratagene
pET-28a (+)	C and N-terminal His•Tag/ T7•Tag [®] configuration.	Novagen
pCR[®] 2.1-TOPO	Amp ^R , Kan ^R , <i>lacZa</i> , T7 promoter, pUC origin of replication.	Invitrogen
pJMK30	<i>Aph-A3</i> Kanamycin resistance cassette, Amp ^R , f1-Ori.	Kindly provided by J. M. Ketley, University of Leicester

Table 2.3 Recombinant plasmids constructed in this study

Recombinant Plasmid	Characteristics
TOPO-<i>cadF</i>-1	pCR [®] 2.1-TOPO with <i>cadF</i> gene from type strain D2686
TOPO-<i>cadF</i>-2	pCR [®] 2.1-TOPO with <i>cadF</i> gene from water isolate SW-OL2
pB-<i>cadF</i>-1	pBluescript KS(+) with inserted <i>cadF</i> gene from strain D2686
pB-<i>cadF</i>-2	pBluescript KS(+) with inserted <i>cadF</i> gene from water isolate SW-OL2
pB-<i>cadF</i>-3	pB- <i>cadF</i> -1 with inserted <i>Aph-A3</i> Kanamycin resistance Cassette at <i>BclI</i> site
pB-<i>cadF</i>-4	pB- <i>cadF</i> -2 with inserted <i>Aph-A3</i> Kanamycin resistance Cassette at <i>BclI</i> site

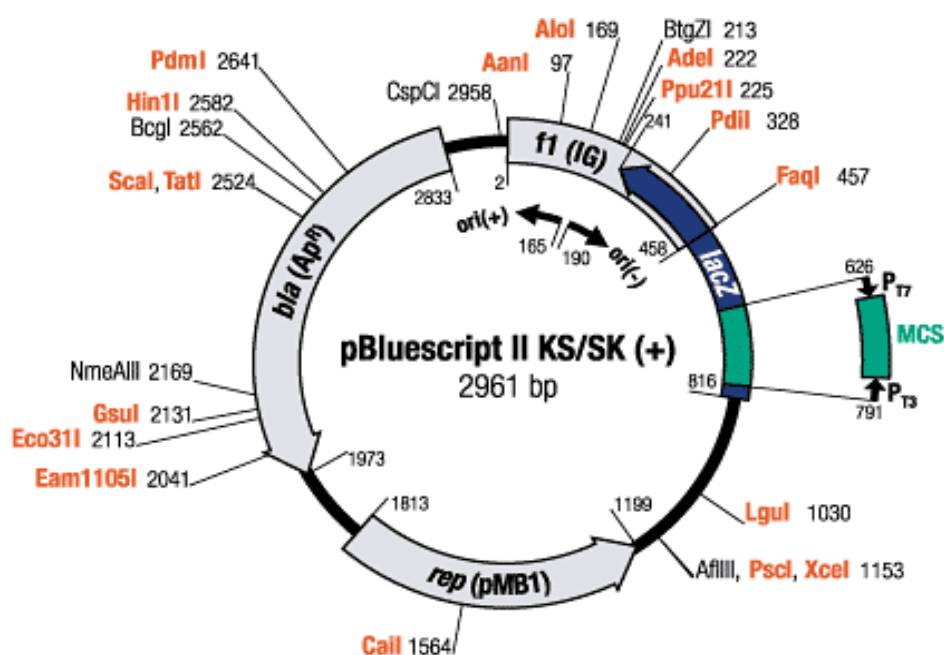


Figure 2.1 Map of pBluescript II KS (+), showing the features and the sequences surrounding the multiple cloning site (MCS). Other restriction sites are labelled to indicate the actual cleavage site.

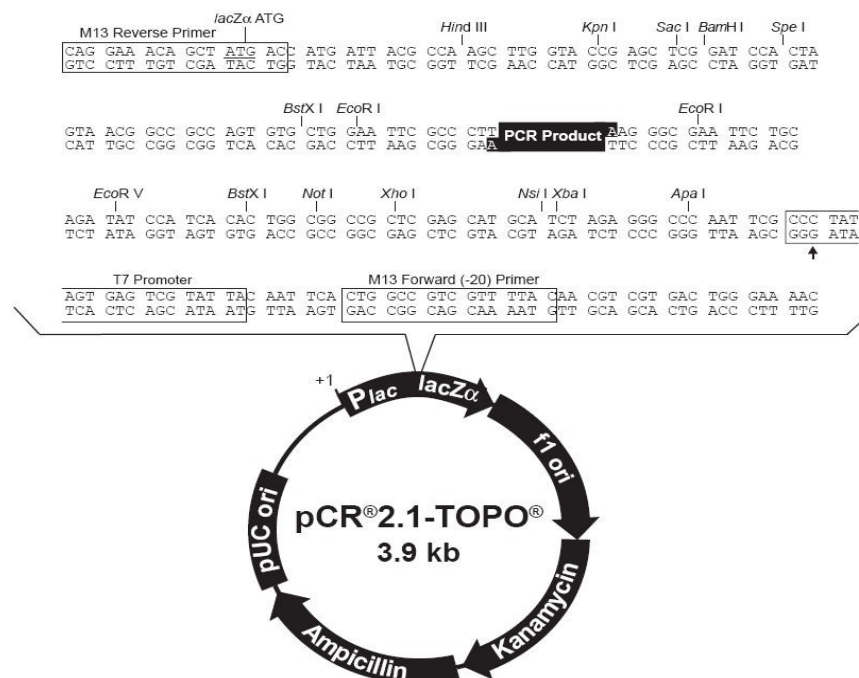


Figure 2.2 Map of pCR® 2.1-TOPO®, showing the features and the sequences surrounding the TOPO® cloning site.

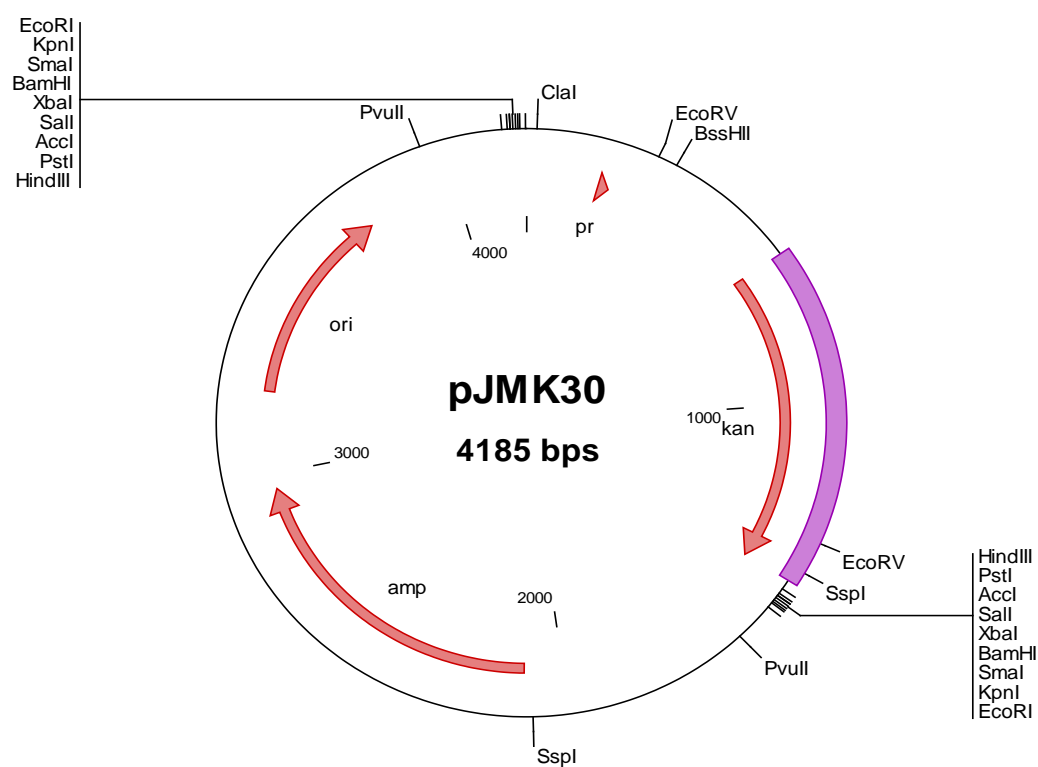


Figure 2.3 Map of pJMK30, showing the features and restriction sites.

2.1.3 Isolation media and bacterial growth conditions

Arcobacter species were grown using *Arcobacter* enrichment basal medium (Oxoid, CM965; Hampshire, UK) with cefoperazone-amphotericin-teicoplanin (CAT) selective supplement (Oxoid, SR174E) as described previously (Atabay *et al.*, 2002). Blood agar was prepared by adding 5% (v/v) defibrinated sheep blood in Blood agar base No.2 (Oxoid, CM271). *Arcobacter* strains were grown microaerobically or aerobically at 28°C for 5-7 days. Gas generating kits (Campylobacter system, O₂ 5% and CO₂ 10%, Oxoid) were used to provide a microaerobic atmosphere.

E. coli strains in this study were cultured in LB medium (containing 1% lactate, 1% tryptone, 0.5% yeast extract and 0.5% NaCl) at 37°C with shaking at 200 rpm overnight. *Bacillus subtilis* was grown for 16 h at 37°C in LB agar or broth.

2.2. Isolation of *Arcobacter* from water samples

2.2.1. Isolation and culture

Water samples were collected from surface waters in sterilised borosilicate glass bottles using aseptic techniques. Containers were immersed in the water source and the bottle moved slowly away from the sampling point to minimise contamination from the hands of the sampler. A small amount of headspace was left in the bottle, which was then capped and chilled for transport back to the laboratory within 2-4 hrs of collection (Table 2.4).

Table 2.4 The sources of water samples from Lothian and Fife regions (Scotland)

No.	Sources of sample	Date of collection	Volume
1	Riccarton Loch	22.04.09	2x 500 ml
2	Duddingston Loch	19.05.09	2x 500 ml
3	Water of Leith (Currie)	14.05.09	2x 500 ml
4	Loch Ore	21.08.09	2x 500 ml
5	Loch Glow	21.08.09	2x 500 ml
6	Loch Gelay	21.08.09	2x 500 ml
7	Loch Portmore	22.08.09	2x 500 ml
9	Loch Leven	22.08.09	2x 500 ml
10	White Adder reservoir	22.08.09	2x 500 ml
11	Baddingsill reservoir	21.08.09	2x 500 ml

The samples were filtered using a 0.45 µm cellulose acetate membrane, which was then immersed in 10 ml phosphate buffered saline (PBS; pH 7.4) with 0.01% Tween-20 (Anonymous, 1995). Five ml of this mixture was then inoculated into *Arcobacter* broth (Oxoid) supplemented with 8.0 mg/l of amphotericin B, 4.0 mg/l of cefoperazone and 8.0 mg/l of teicoplanin (ACT, Oxoid) and incubated microaerobically at 28°C for 5 days. A gas generating kit (Oxoid) was used to provide a microaerobic environment. The resulting broth cultures were then streaked onto four solid media including *Arcobacter* agar, sheep blood agar, nutrient agar, and MacConkey agar. All plates were incubated aerobically and microaerobically at 28°C for 2-5 days after which time the cultures were examined for phenotypic characteristics (e.g. white or grey round colonies). Colonies typical of *Arcobacter* were subcultured on *Arcobacter* agar, blood agar, and nutrient agar. The isolates were kept on blood agar or nutrient agar for identification by phenotypic and genotypic means.

2.2.2. Phenotypic characteristics

Putative *Arcobacter* colonies were evaluated by Gram staining and oxidase tests as proposed for *Campylobacter* (Ataby *et al.*, 2006; Ursing *et al.*, 1994). Following this, potential *Arcobacter* strains were purified by streaking on blood agar and phenotypically characterised using the biochemical tests listed in Table 1.3. All tests were carried out according to recommended methods described previously (Atabay *et al.*, 2006; Vandamme *et al.*, 2005; On *et al.*, 1996) and reference strains of *A. butzleri* (D2686), *A. cryaerophilus* (A169/B) and *A. skirrowii* (449/80) from the National Collection of Type Culture (NCTC) were used as positive controls throughout these studies.

2.2.3. Extraction of genomic DNA

Genomic DNA was isolated from 1 ml of broth cultures using a DNeasy Blood and Tissue kit, QIAgen. Briefly, cultures were harvested once they had reached OD₆₀₀ of 0.5-0.6 (late exponential growth phase; approximately 5.0×10^8 cells) by centrifugation at $6000 \times g$ for 20 min and the DNA extracted as instructed by the manufacturer; the DNA was stored at -20°C until use. An alternative method, described by Fera *et al.* (2004), was also used in some protocols in this project. Briefly, bacterial genomic DNA was prepared by resuspending a cell pellet (5.0×10^8 cells) in 50µl of TE buffer (1 mM Tris/ HCl pH8.0 and 0.5 mM EDTA). This mixture was boiled for 5 min at 100°C and used directly as template DNA for PCR and other procedures.

2.2.4. Preparation of plasmid DNA

Bacterial cells were grown in 5 ml LB (Luria-Bertani, containing 10 g Tryptone, 5 g yeast extract and 10 g NaCl per litre) with selective antibiotics for *E. coli* or *Arcobacter* broth (supplemented with 15% Casamino Acids, BIO 101) for all *Arcobacter* species at 28°C, overnight (for *E. coli*) or 3-5 days (for *Arcobacter spp.*) with shaking at 200 rpm. A Mini-prep QIAquick plasmid extraction kit (QIAgen) was used to extract plasmid DNA from bacterial cultures.

2.2.5. RNA extraction

An RNeasy mini kit (Qiagen) was used for RNA purification and the manufacturer's instructions were followed. RNA was isolated from 0.5-1.0 ml of culture containing $5.0\text{--}7.5 \times 10^8$ cells. Purified bacterial RNA was protected by adding 1 ml of 'RNA Protect' (Qiagen) to the culture sample.

2.2.6. Phenol: chloroform extraction

To remove protein contaminants from DNA-containing solutions, the solutions were mixed with an equal volume of phenol-chloroform (1 volume phenol and 1 volume chloroform/iso-amyl alcohol {24:1, v/v}, Sigma) in a 1.5 ml microfuge tube. The mixture was vortexed, and then centrifuged for 5 min at 13,000 rpm; the upper aqueous layer (containing the DNA) was transferred to a fresh 1.5 ml Eppendorf tube and mixed with 1 volume of chloroform to remove residual phenol with the aqueous phase being separated from the organic phase by centrifugation for 10 min at 13,000 rpm. The top phase was removed into a new tube and the nucleic acid concentrated by ethanol precipitation.

2.2.7. Polyethylene glycol precipitation (PEG) of DNA

Occasionally DNA was precipitated using polyethylene glycol as described by Lis (1980), with modifications by Paithankar and Prasad (1991).

2.2.8. Ethanol precipitation

DNA was precipitated from solutions by the addition of 0.1 volume of 3M sodium acetate pH 5.2 and two volumes of 96% (v/v) ethanol. The mixture was incubated for 30 min at -20°C and then centrifuged at 13000 rpm for 15 min. The supernatant was removed and the pellet washed with 1 ml of 70% ethanol, followed by centrifugation for 10 min at 13000 rpm. The pellet was dried and resuspended in dH_2O or TE buffer (1 mM Tris/ HCl pH8.0 and 0.5 mM EDTA) to the desired volume.

2.2.9. Measurement of DNA and RNA concentration

DNA and RNA concentration was measured using a spectrophotometer set at a wavelength of 260nm. The concentration of DNA and RNA was then calculated based on the following rules:

1 O.D. at 260 nm for double-stranded DNA = 50 ng/μl of dsDNA

1 O.D. at 260 nm for RNA molecules = 40 ng/μl of RNA

Typically, the DNA or RNA solutions were diluted 1:200 and 1:400 (for DNA and RNA respectively), so the following equations were used to determine the concentrations of DNA and RNA:

$A_{260} \times 50 \text{ ng/}\mu\text{l} \times \text{dilution factor for DNA concentration}$

$A_{260} \times 40 \text{ ng/}\mu\text{l} \times \text{dilution factor for RNA concentration}$

2.2.10. Gel purification of DNA fragments

A QIAquick gel extraction kit (Qiagen) was used to purify DNA fragments from agarose gels. The DNA band of interest was excised from a 1% (w/v) agarose gel in 1xTAE buffer (Appendix) with a sharp, clean scalpel and placed in a pre-weighed Eppendorf tube. After weighing the manufacturer's instructions were followed.

2.2.11. Dephosphorylation of DNA by alkaline phosphatase

In order to prevent recirculation of linearised plasmid DNA, 1U of shrimp alkaline phosphatase (EF0511, Fermentas) was added to the digest with 1X SAP buffer (Fermentas) and incubated at 37°C for 30 min, followed by 15 min at 65°C to inactivate the alkaline phosphatase.

2.2.12. Polymerase chain reaction (PCR)

2.2.12.1. Oligonucleotide primers

All primers were designed by using primer3 software and were designed to contain sufficient G/C % to enhance primer binding to the template DNA. Primers were synthesised by MWG Biotech and all primers were stored at -20°C at a concentration of 100 pmol/μl. The sequences of the primers used are listed in Table 2.5.

Primers for RT-PCR were designed based on following initial considerations: amplification product size around 150-200 bp, primer size 20 ± 2 bp, G/C content 50% and primer T_m $58 \pm 2^\circ\text{C}$ (Table 2.6). BLAST searches were also performed against other bacterial genomes to determine the specificity of these primers.

Table 2.5 The primers for amplification, cloning and sequencing

Primer name	Sequence (5'→3')	T _m	Product size	Use or gene target	Reference	Source
1492R	GGCTTACCTTGTTACGACTT	60°C ^a	----	Sequencing of 16S rDNA	Lane <i>et al.</i> , 1991	MWG-Biotech
F27	AGAGTTTGATC(A/C)TGGCTCAG					
BUTZ	CCTGGACTTGACATAGTAAGAATGA	58.5°C ^a	401 bp	Amplification of 16S rDNA of <i>A. butzleri</i>	Houf <i>et al.</i> , 2000	
ARCO1	CGTATTCACCGTAGCATAGC					
CRY1	TGCTGGAGCGGATAGAAGTA	57.3°C ^a	257 bp	Amplification of 23S rDNA of <i>A. cryaerophilus</i>		
CRY2	AACAACCTACGTCCTTCGAC					
SKIR	GGCGATTTACTGGAACACA	60°C ^a	641 bp	Amplification of 16S rDNA of <i>A. skirrowii</i>		
ARCO2	CGTATTCACCGTAGCATAGC					
1R(RADP)	GGTGC GGAA	40°C ^a	----	RAPD-PCR	Williams <i>et al.</i> , 1990;	
OPAU-01	GGGATGGAAC		----		Welsh and	
OPAU-02	CCAACCCGCA		----		McClelland, 1990;	
OPAU-03	ACGAAACGGG		----		Caetano <i>et al.</i> , 1991ab;	
OPAU-04	GGCTTCTGTC		----		Munthali <i>et al.</i> , 1992	
OPAU-17	GACCGCTTGT		----			
KancasF	AAGCTACCAAGACGAAGAGG	60°C ^a	----	Kanamycin resistance cassette	Konkel <i>et al.</i> , 1999a;	
KancasR	GCTCGACATACTGTTCTTCC			<i>aphA-3</i> gene	Malgorzatal <i>et al.</i> , 2007	
M13-F	GTAAAACGACGGCCAGTGAA	56°C ^a	----	Screening test for cloning with plasmid containing M13 sequences	Invitrogen	
M13-R	CCTTTGTCGATACTGGTACT					
<i>CadF</i> -FP1	ATGAAAAAAGTATTATTATCA	50°C ^b	----	<i>cadF</i> gene	This study	
<i>CadF</i> -RP1	TTATTTAACCATTACAGCTT					
<i>CadF3</i> Fwd	CAATAATAGGAGAAGCAATT	50°C ^b		<i>cadF</i> gene	This study	
<i>CadF3</i> Rev	TGTTACAGTTAAAATGTCAA					

^a; T_m values were obtained from the previous studies as detailed with each reference.

^b; T_m values were estimated by using Primer3 primer design programme.

Table 2.6 The primers for RT-PCR and Probes

Gene target	Sequences (5'→3')	Primer length (bp)	T _m (°C)	Amplicon length (bp)	Source
<i>cadF</i>	Fw-CGGTGGAGTTTAACTGAAGG	21bp	58.75	150bp	MWG-Biotech
	Rv-TTTCCTTTTCCATCAACGTCTT	22bp	59.98		
<i>ciaB</i>	FW-GTGGGACGACCAGCACTATT	20bp	60.00	150bp	
	Rv-TGGTTTAGCACGGCTAGTTTG	21bp	60.30		
<i>flaA</i>	Fw-CTGCAAAAGGTGGATTGTCTT	21bp	59.23	150bp	
	Rv-CAGTTGCACCAGCTGACATT	20bp	59.90		
<i>flaB</i>	Fw-GCAAGAACTGCATTAGGTGGA	21bp	60.26	155bp	
	Rv-GTTGCACCAGCTGACATTTT	20bp	58.78		
<i>pldA</i>	Fw-AAAATCTTTTTGGCAAGTAGCAA	23bp	58.75	150bp	
	Rv-TTCTGCATCTTTTCCATTTGA	21bp	59.98		
<i>glnA</i>	Fw-TGGGAAAATTTGTAAATAACAC	22bp	56.75	168bp	
	Rev-CATCAAAAGGCATTCCATTATC	22bp	60.20		

2.2.12.2. PCR reaction

PCR amplification was carried out in a 50 µl reaction volume containing, 1.25 mM dNTP mix (Bioline), 1.5 mM MgCl₂ (Bioline), 1X buffer (Bioline), 10 pmol of each primer (forward and reverse), and 0.1-1.0 µg of template DNA and the PCR reaction mixture was made up to 50 µl with sterile millipure water. The PCR reaction mix was heated at 95°C in a PCR machine (Icycler, Bio-Rad) for 5 min before adding 1.0 µl of Taq DNA polymerase (5U, BioLabs), followed by 35 cycles of PCR reaction with denaturation at 95°C for 45 sec, annealing for 30 sec and elongation at 72°C for 30 sec. The annealing temperature was determined as $T_m - 5^\circ\text{C}$, where T_m is the melting temperature of the primers. Additionally, a final extension was done at 72°C for 10 min and the reaction mix was stored at 4°C until used.

2.2.13. Agarose gel electrophoresis

Agarose gels contained 1% (w/v) agarose in 1×TAE buffer (Appendix). Agarose was dissolved in TAE buffer and ethidium bromide was added to the gel (final concentration 0.5µg/ml). Prior to electrophoresis all samples containing DNA were mixed with 5×DNA loading buffer (BioLabs) at a ratio 1:3 and loaded on the gel. 5 µl of Hyperladder (Bioline) was also loaded and run alongside the samples. The gel was run in 1×TAE buffer at 70-100V for 30-60 mins before viewing the bands on a UV-Light (Labimage, BIORAD).

2.2.14. Species specific amplification of 16S rDNA

Six primers were used depending on the 16S and 23S rDNA sequences of several *Arcobacter* species (*A. butzleri*, *A. cryaerophilus* and *A. skirrowii*) used in this study (BUTZ, ARCO1, CRY1, CRY2, SKIR and ARCO2 respectively), designed by Houf *et al.* (2000). All primers used in this project are shown in Table 2.5.

2.2.15. Genomic fingerprinting

2.2.15.1. RAPD-PCR

Random amplification of polymorphic DNA (RAPD) is a rapid and simple method for genotyping different bacterial strains (Hadrys *et al.*, 1992). The PCR profiles were produced using six different RAPD-primers described in Table 2.5. RAPD-PCR conditions were as described in Section 2.2.12.2 with some modifications as suggested by Houf *et al.* (2002). The cycling parameters were as follows: initial denaturing at 94°C for 5 min and 45 cycles of denaturation at 95°C for 30s, annealing at 36°C for 1 min, and extension at 72°C for 2 min. Genomic DNA was used as template and one type strain (D2686) used as positive control.

2.2.16. DNA sequencing and sequence analysis

All DNA samples were sequenced by the GenePool group of Edinburgh University and sent as mixture containing 1-5 µg of purified DNA and 1 µl (2.3 pmol/µl) of each primer and analysis of sequences was carried out using free software, ClustalW2 EBI (1) or Blast2tree (2).

(1): (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

(2): (<http://bioinfo.unice.fr/blast/blast2tree/blast2tree.php>)

2.3. Pathogenicity analysis of reference strains and water isolates

2.3.1. Qualitative characterisation of extracellular enzyme production

2.3.1.1. Plate assay of caseinase activity

The ability of strains to produce caseinase (protease) was examined by growing them on double strength TSA (Appendix) agar plates (Oxoid) supplemented with 4% sterile skimmed milk (Zhang, 2001). 100 µl of bacterial suspension (adjusted to an OD₆₀₀ of 0.5-0.6, approximately 5x10⁸ CFU/ml) of *Arcobacter* strains was spotted onto the plates and incubated at 28°C for 5-7 days; the clear zones of hydrolysis around the resulting bacterial colonies were examined and measured.

2.3.1.2. Plate assay of gelatinase activity

The ability of strains to produce gelatinase was determined by streaking cultures (approximately, 5×10^8 CFU/ml) onto TSA supplemented with 0.5% (w/v) gelatine (Zhang, 2001; Loghothetis and Austin, 1996). After 5 days incubation at 28°C, the appropriate volume of saturated ammonium sulphate solution was dispensed onto the plates and the clear zones around the bacteria confirmed that gelatin had been hydrolysed.

2.3.1.3. Plate assay of elastase activity assay

Elastase production was evaluated for all strains using a two-layered agar procedure described by Hasan *et al.* (1992). First, Columbia agar base (CAB; Oxoid) was used to prepare a base plate before a further 10 ml CAB with 1% sodium chloride and 0.3% elastin added was over-layered on top. The CAB agar was swirled gently but continuously while the top layer was poured, to obtain a homogenous suspension. 100 µl of *Arcobacter* suspension (the suspension was adjusted to an OD₆₀₀ of 0.5-0.6, approximately, 5.0×10^8 CFU/ml) were spotted onto the plates and incubated for 20-28 days at 22°C. Detection of elastase activity was indicated by zones of clearing around the colonies.

2.3.1.4. Plate assay of phospholipase and lipase activity

Phospholipase and lipase activities were tested by inoculation of bacterial culture ($4-5.0 \times 10^8$ CFU/ml) onto TSA supplemented with 1% egg yolk emulsion (Sigma) and 1% Tween 80 (Sigma). After incubation of the plates at 28°C for 5-7 days, the diameter of the opalescent precipitation zone (an indicator of extracellular phospholipase activity) was recorded (Liu *et al.*, 1996).

2.3.1.5. Plate assay of haemolytic activity

Haemolysin activities of *Arcobacter* cultures were examined by preparing, individually, agar plates containing 5% of red blood cells from various animals (sheep, horse, cattle, salmon and chicken). Bacterial cultures ($4-5.0 \times 10^8$ CFU/ml) were inoculated onto the

blood agar plate being tested, and the presence and size of zones of clearing around the colony following incubation at 28°C for 5 days was recorded (Honda *et al.*, 1988).

2.3.2. Characteristics of the ECP, WCP and OMP proteins

2.3.2.1. Preparation of whole cell proteins (WCPs)

Whole cell protein extracts were prepared by harvesting cells from 20 ml of 3 days-old bacterial growing culture (0.6-0.7 OD at 595nm) by centrifugation at $8000 \times g$ for 20 min and the pelleted material recovered into 10 ml of phosphate buffered saline. The absorbance was adjusted to give a density of between 0.6 and 0.7 at 595 nm and 2 x 1.5 ml volumes of the suspension were centrifuged for 3 minutes at $14,000 \times g$ at 4°C. The supernatants were discarded and each pellet was resuspended in 30 µl volume of SDS-PAGE sample buffer (Appendix) before being pooled to give a final sample volume of 60 µl. The suspension was boiled for 5 min, and stored at -20°C until required (Atabay *et al.*, 2003; Zhang, 2001).

2.3.2.2. Preparation of outer membrane proteins (OMPs)

Outer membrane proteins were separated from *Arcobacter* strains and water isolates as described by Poxton (1979) and modified by Zhang (2001). Bacterial culture was harvested from three plates into 10 ml PBS, then they were collected after centrifugation at $4000 \times g$ for 15 min at 4°C and suspended again in 10 ml PBS, followed by re-centrifugation, and resuspension in 2 ml of PBS supplemented with 10 mM EDTA and 2 mM phenylmethanesulphonyl fluoride (Sigma) as protease inhibitor. After incubation in a water bath for 30 min at 45°C, the suspension was subjected to ultrasonic treatment for 2 min to release OMPs, and centrifuged at $13,000 \times g$ at 4°C for 15 min. The supernatants were collected and stored at -20°C, until required.

2.3.2.3. Preparation of extracellular proteins (ECPs)

The method used was that described by Song *et al.* (2004) with some modifications. Bacteria were grown in 40 ml of *Arcobacter* broth and harvested by centrifuging at $8000 \times g$ for 10 min. The cells were washed twice in 10 ml of PBS (pH 7.4) and centrifuged again at $4000 \times g$ for 10 minutes. The washed cells were resuspended in 10 ml of PBS and stored at -20°C . The ECPs were recovered by filtering the culture supernatants through a $0.22 \mu\text{m}$ filter (Millipore) and precipitating by adding two volumes of 10% (w/v) trichloroacetic acid (Sigma) with overnight incubation at 4°C . Precipitated proteins were then centrifuged for 20 minutes at $7000 \times g$. The pellets were resuspended with 1.5 ml of 90% cold acetone, centrifuged at $15,000 \times g$ for 5 minutes and dried. Finally, the pellets were resuspended in 0.5 ml of PBS and stored at -20°C .

2.3.2.4. Determination of protein concentration

Total protein concentration was estimated using the Bradford assay (Bradford, 1976). The measurements were carried out according to the manufacture's instructions (Bradford Reagent, Sigma).

2.3.2.5. SDS-PAGE analysis of proteins

SDS-PAGE analysis was carried out using the method described by Laemmli (1970) with some modifications. Proteins were separated in 12% SDS-acrylamide separating (resolving) gel (Appendix) over-layered with a 4% stacking gel (Appendix). Samples were added to the gels at a volume of $10 \mu\text{l}$ per well. The gels were subjected to electrophoresis under constant voltage (200V) for 1.5 h on SDS-PAGE apparatus (ATTO Corporation, Japan) using Tris-glycine running buffer (Appendix). The gel was stained in an appropriate volume of Coomassie Brilliant Blue solution (Sigma) for 1 h at 37°C . The gel was washed and destained by incubation in an appropriate volume of destain solution (Appendix) overnight at room temperature with shaking. Proteins were detected as blue-stained bands against a clear background by using Labimage software (BIORAD).

2.3.3. Quantitative characterisation of the ECPs of all strains

2.3.3.1. Caseinase activity assay

Caseinase activity of ECPs was determined using azocasein (Sigma) as substrate (Austin *et al.*, 1998). Fifty μl of ECPs were incubated with 450 μl of 1% (w/v) azocasein in 1.0M PBS (pH 7.2) at 28°C for 30 minutes, PBS was used as a reagent blank instead of ECPs. The reaction was stopped by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid (Sigma). After 30 minutes, the precipitate was pelleted by centrifugation at $14,000 \times g$ for 5 minutes at 4°C, and a 0.5 ml volume of the supernatant added to 0.5 ml of 1M NaOH. Released azodye was measured as A_{450} on a UV spectrophotometer (Bio-Rad) against a reagent blank. The assay was performed in triplicate. One caseinase unit was defined as an increase of 0.001 in A_{450} under the assay condition.

2.3.3.2. Gelatinase activity assay

The gelatinase activity was determined by using the method of Zhang (2001) modified as follows: 50 μl of ECPs were incubated with 450 μl of 0.8% (w/v) gelatine (Oxoid) in 0.1M PBS (pH 7.2) at 28°C for 30 min, the reaction was stopped by the addition of 1.5 ml of 30% (w/v) trichloroacetic acid. After 30 min, the precipitate was pelleted by centrifugation at $3000 \times g$ for 10 min at 4°C. The supernatant was measured as A_{280} against a reagent blank (PBS). One unit of gelatinase was defined as an increase of 0.001 under the assay condition.

2.3.3.3. Phospholipase and lipase activity assays

Phospholipase and lipase activities were determined using 20 μl of ECPs, which were pipetted into 4-mm diameter wells cut in 1% agarose prepared in PBS (pH 7.2) supplemented with 1% (v/v) egg yolk emulsion or 1% (v/v) Tween 80 (Liu *et al.*, 1999). The plates were incubated for 48 h at 28°C, whereupon the presence and size of the opalescent zone around each well was recorded.

2.3.3.4. Haemolytic activity assay

Haemolytic activities of ECPs were determined as described by Anju *et al.* (2010) with some modifications, against human, sheep, rabbit, horse, salmon and chicken erythrocytes (Sigma and Seralab). The appropriate volumes (approximately, 200 µl) of ECPs were pipetted into 4-mm diameter wells cut in blood agar with 5% of red blood cells from each source. All plates were incubated at 28°C for 48 h and the haemolytic activity was observed in the form of clearance zones.

2.3.4. Identification and cloning of *cadF* gene

2.3.4.1. Medium and antibiotics used for growth of *E. coli* strains

Luria-Bertani Broth (Appendix), SOC medium (Appendix) and Nutrient Broth and agar (Oxoid) were used for growth of all *E. coli* strains. All media were autoclaved at 121°C for 15 minutes. Appropriate antibiotics were added to all these media according to resistance genes within plasmids or genome of bacterial strains. In most cases, 50-100 µg/ml (w/v) of ampicillin (Oxoid) or 50-100 µg/ml (w/v) of kanamycin (Oxoid) were used in this study.

2.3.4.2. Growth of *E. coli*

All *E. coli* strains were stored at -70°C in LB broth containing 15 (v/v) glycerol before streaking them on LB agar containing appropriate antibiotics. The plates were incubated overnight at 37°C and an individual colony was picked and transferred into 10 ml LB broth. The culture was incubated overnight with shaking at 37°C. Then 1 ml of each culture was transferred to 100 ml LB Broth and incubated at 37°C until reaching the desired value for cell harvest at OD₆₀₀.

2.3.4.3. Amplification of the *cadF* gene for cloning and sequencing

The *cadF* gene was amplified by PCR as described in Section 2.2.12 and the primers were designed as described in Section 2.2.12.1 (**Table 2.5**) , using genomic DNA of

Arcobacter strains as a template. The PCR products were purified using the PEG protocol as described in Section 2.2.7.

2.3.4.4. Restriction enzyme digestion

Many different restriction enzymes were used to digest different DNA fragments and plasmids according to the purpose of digestion. The digestion was usually carried out in a 20-50 µl final volume containing 0.5-2 µg of undigested DNA, the appropriate 1x reaction buffer (each enzyme has particular buffer) and for each 1 µg of DNA 10 units of restriction endonuclease was needed. All reactions were incubated for 1-3 h at the appropriate incubation temperature according to each enzyme.

2.3.4.5. Ligation

DNA fragments were ligated in a total volume 10-20 µl according to the molecular ratio of linear insert (target fragment or gene) and linear vector (plasmid) as follows:

$$\text{Amount of insert (ng)} = \frac{\text{Amount of vector (ng)} \times \text{kb size of insert}}{\text{Size of vector (kb)}} \times \text{molar ratio of insert/vector}$$

In most cases either a 1:3 or 1:5 molar ration of vector/insert worked well. T4 DNA ligase was used as described in the manufacturer's protocol (Fermentas). Vector and insert were added together to a sterile microcentrifuge tube with 1-2 µl 10X ligation buffer (0.4 mM Tris-HCl, 0.1 mM MgCl₂, 0.1 M DTT, 5 mM ATP, final concentration at 1X buffer) and 5 units of T4 DNA ligase. The final volume of the ligation reaction was 10-20 µl; samples were incubated at 37°C for 1-4 h at room temperature, followed by heating the mixture at 75°C for 15 min.

2.3.4.6. Preparation of chemically competent cells

The method described by Inoue *et al.* (1991) was used to prepare the chemically competent cells (*E. coli* strains). 5 ml of Luria Broth (LB, Appendix) containing 100 µl of 1M MgSO₄ was inoculated with a single colony of the *E. coli* strain XL1-Blue MRF

and incubated overnight at 37°C with shaking until OD₆₀₀ increased to a certain value (0.5 to 1.0) for cell harvest. This culture was used to inoculate 250 ml of LB containing 5 ml of 1M MgSO₄ and incubated at 28°C with good aeration by rotation at 250 rpm until the OD₆₀₀ reached 0.4 to 0.6. The culture was transferred into a sterile bottle and placed on ice for 10 min before being centrifuged at 3000 rpm for 10 min at 4°C. The pellets were resuspended gently in 80 ml cold transformation buffer (Appendix) and centrifuged at 3000 rpm for 10 min at 4°C, then resuspended in 20 ml cold transformation buffer with 1.5 ml dimethyl sulfoxide (DMSO). The suspension was placed on ice for 10 min before being dispensed in 200 µl aliquots into cold sterile microfuge tubes and stored at -70°C until required. Cells were thawed on ice before being used for transformation.

2.3.4.7. Chemical transformation

1-5 µl of plasmid was added to 200 µl of competent cells in a 1.5 ml microcentrifuge tube and mixed very gently. The cells were placed on ice for 30 minutes, heat-shocked for 45 sec at 42°C in a water bath, then placed on ice for 1-2 minutes. 1 ml of LB was added to the samples (without any addition of antibiotics) and incubated at 37°C for 1h. The cells were harvested by centrifugation at 6000 rpm for one minute. The supernatant was removed and the cells were resuspended in 100 µl LB, spread onto LB plates supplemented with appropriate antibiotics, and incubated at 37°C overnight. Stock solution of X-gal (100 mM) was prepared in dimethylformamide (Sigma), while IPTG (100 mM) was prepared in distilled water. 5 µl of X-gal and 25 µl of IPTG were spread onto the surface of an LB-Agar plate containing appropriate antibiotics. 50 µl of bacterial sample was plated out onto the medium and the plate incubated at 37°C overnight. The plate was subsequently screened for white colonies which contained plasmid with insert DNA.

2.3.4.8. Electrotransformation

Competent *Arcobacter* cells for electro-transformation were prepared as follows. A 10ml pre-culture in *Arcobacter* medium was incubated at 28°C for 5 days and 4 ml of

pre-culture was diluted into 200-ml of fresh *Arcobacter* broth pre-warmed at 28°C and incubated with good aeration by rotation at 250 rpm. When OD (600nm) reached 0.5-0.6, the culture was chilled on ice for 20 min. The bacterial cells were transferred to sterile ice-cold centrifuge bottles and centrifuged at 5,000 rpm for 10 min at 4°C. The cell pellets were suspended in 100 ml ice cold sterile water and centrifuged at 5,000 rpm for 10 min at 4°C. Suspended cell pellets in 40 ml of ice cold water were transferred to a sterile 50 ml conical centrifuge tube. The supernatant was discarded and the pellets resuspended in 5 ml of sterile ice-cold water. 200 µl volumes of cell suspension were transferred into 1.5 ml sterile micro-centrifuge tubes (cell concentration approximately 5×10^8 CFU/ml). 10% glycerol (molecular biology grade) was added, and the electro-competent cells of *Arcobacter* were stored at -70°C.

A 2 mm-gap electroporation cuvette was placed on ice and 1 µl of plasmid was mixed gently with 100 µl of bacterial suspension (the suspension was adjusted to an OD₆₀₀ of 0.5-0.6, approximately 5×10^8 CFU/ml) of *Arcobacter* strains and left on ice 1 min. The mixture was transferred to the 2 mm electroporation cuvette (EQUIBIO), tapped to the bottom and a tissue paper was used to remove residual water from around the cuvette. The cuvette was placed in the cuvette tray and applied to the electroporator (Eppendorf) pre-set as follows: Resistance in Pulse Controller, 200Ω; Capacitance in Capacitance Extender, 250 µFD; Capacitance in Gene Pulser, 25 µFD; Voltage, 2.5 KV. The time constant was 4.0 to 4.7 milliseconds. 1 ml of LB medium was immediately added, aseptically, to the mixture in the cuvette; the suspension was mixed with a pipette then transferred to a microfuge tube and incubated at 28°C overnight with shaking. 50 µl of the suspension was spread out onto nutrient agar plates with appropriate antibiotics and incubated at 28°C for 5-7 days. Individual colonies were selected and tested as described in Section 2.3.4.9 for identification purposes.

2.3.4.9. Screening of colonies

Each target colony was picked and resuspended in 20 µl of millipore dH₂O. The suspension was heated at 100°C for 10 min, transferred to ice for 5 min, and then centrifuged at $14,000 \times g$ for 2 min. 1 µl of the supernatant was used as template for PCR as described in Section 2.2.12 with appropriate primers to examine whether the

desirable plasmid and other DNA fragments had been cloned into the vector. Restriction endonuclease digestion was carried out as described in Section 2.3.4.4. Sequencing of target DNA fragments was also used to further identify the target DNA fragments as described in Section 2.2.16, using appropriate primers as detailed in Table 2.5.

2.3.5. Tissue Culture

2.3.5.1. Culture of INT-407 cells

A stock culture of INT-407 cells (derived from human embryonic intestine, ATCC CCL 6) was obtained from European Collection of Cell Cultures (Health Protection Agency Culture Collection). INT-407 cells were cultured in 75 cm² flasks (Nunc), in Eagle minimal essential medium (EMEM, Sigma) supplemented with 10% (v/v) foetal bovine serum (Sigma), 100 µg/ml streptomycin, 100 units/ml penicillin G (Sigma), 1% (v/v) non-essential amino acids (Sigma) and 1% (v/v) of 200 mM L-Glutamine (Sigma) at 37°C in a humidified, 5% CO₂ incubator. The growth and the confluency of the INT-407 cells were determined by microscopic examination and confluent monolayers reached 70-80% on day 5-7 ($\approx 10^5$ - 10^7 cells cm⁻²). Confluent INT-407 monolayers were trypsinized using trypsin-EDTA solution (1X, Sigma), diluted 1/40 in fresh EMEM and then seeded into wells of a 24-well tissue culture plate before being incubated at 37°C in a humidified, 5% CO₂ and 95% air atmosphere. For adhesion and infection assays, the monolayers were washed four times with warm plain EMEM (without antibiotics or supplements) or PBS (pH 7.2). This was carried out at least 1 h before the assays were started.

2.3.5.2. Adherence assay

The first adherence assay was performed as described by Kusters *et al.* (1993) with some modifications. A 24-well tissue culture tray (Nunc) was seeded with INT 407 cells (2×10^5 cm⁻²) per well and incubated at 37°C in a humidified, 5% CO₂ incubator till the confluency reached 70-80%. The bacteria were harvested in EMEM supplemented with 1% PBS and washed twice and the optical density at 600 nm (OD₆₀₀) of the bacterial suspension was adjusted to 0.4-0.5 (≈ 2 - 5×10^8 CFU/ml). Tenfold serial

dilutions of the initial suspension were used to inoculate INT 407 cells as described by Monteville *et al.* (2002). Briefly, the INT 407 cells were washed with EMEM or PBS (pH 7.2) and 1 ml of a bacterial suspension was added to each well. After 3 h of incubation, the non-adherent cells were removed by rinsing with PBS (pH 7.2). The INT 407 cells were lysed with a 0.1% Triton X-100 solution in PBS for 30 min at 37 °C. The suspensions were serially diluted 10-fold, and the number of viable adherent bacteria was determined by counting the resultant colonies on *Arcobacter* agar plates with supplement (ATC, Sigma).

The adherence assay was also carried out as described by Carbone *et al.* (2003). 1 ml of INT-407 cells ($\approx 2 \times 10^5$ cells/ml) in growth medium was placed in 4-chamber 16 mm chamber slide Tissue Culture Slides (Thomas Scientific or Nunc-lab-Tek Chamber slide system) at 37°C in a 5% CO₂ incubator till the confluent monolayers reached 70-80%. The INT-407 cells were then washed three times with plain EMEM before 1 ml of bacterial suspension ($\approx 2 \times 10^8$ cells) was added to each chamber and the slides incubated at 37°C for 3 h in a 5% CO₂ atmosphere. Each chamber was then washed four times with PBS (pH 7.2) and the plastic media chambers were removed. Bacteria bound to INT-407 cells on the plastic coverslip were fixed with 99% methanol, stained with 10% Giemsa stain and examined microscopically under oil immersion. *E. coli* was used as positive control and the adhesion index was determined as the number of bacteria adhering to each of 40 INT-407 cells. Bacteria with an adhesion index of 10 or less were regarded as non-adhesive and the bacteria having an adhesion index of 40 or greater were considered as strongly adhesive.

2.3.5.3. Invasion assay and extraction of bacterial RNA from Tissue Culture

For invasion assay, 2 ml of INT-407 cells were seeded into each well of a 6-well tissue culture tray (Nunc) at about 1×10^5 cells per well in EMEM–10% PBS without antibiotics. 1 ml of bacterial suspension ($\approx 2 \times 10^8$ cells) was added per well, incubated at 37°C for 6 h in a 5% CO₂ incubator and then washed twice with EMEM or PBS (pH 7.2). Infected INT-407 cells were lysed for 30 min on ice, in enough lysis buffer (0.1% SDS, 1% phenol pH 4.3 (Sigma), 19% ethanol in H₂O) to cover the cell layer. Lysates

were collected in cold Eppendorf tubes and the pellets were collected by centrifugation ($14,000 \times g$, 10 min, 4°C). The pellets were resuspended with PBS (pH 7.2), two volumes of RNA Protect Bacterial Reagent (Qiagen) were added and the pellets were collected again by centrifugation ($14,000 \times g$, 10 min, 4°C) and stored at -20°C until RNA was extracted as described in Section 2.2.5.

2.3.6. DNA and RNA Slot Blot

2.3.6.1. Preparation of DIG-labelled probes as dUTP PCR products

DIG-labelled DNA probes were synthesized by standard PCR amplification of genomic DNA of *Arcobacter butzleri* strain D2686, with the primers for each target gene (Table 2.5) using 2 μl of Digoxigenin-11-dUTP with Taq DNA polymerase in 50 μl of the PCR mixture as described Section 2.2.12. About 25 ng/ml of each probe was used in RNA and DNA slot blot.

2.3.6.2. Examination of DIG-labelled probes

5 μl of PCR amplified DIG-labelled DNA probe (25 ng/ml) was diluted to 100 μl with dH_2O and an appropriate volume was applied to a piece of dry Nylon membrane (Roche). The DNA probes were cross-linked to the membrane by irradiating both sides of the membrane under 1.2 kJ/m^2 for 1 min with UV light in an ultraviolet cross-linker (Ultra Lum, UVC-508). Fluorescence was detected as described in Sections 2.3.6.5 and 2.3.6.6.

2.3.6.3. DNA slot blot

To identify the putative virulence genes involved in this project, slot blotting of DNA was carried out using DIG-labelled DNA probes prepared as described in the previous sections. Slot blotting was carried out in a Minifold II System (Schleicher & Schuell), which was assembled based on the manufacturer's directions using transparent plastic film, silicone spacer gaskets, a vacuum filter support and sample well plate.

Two pieces of filter paper (Whatman, 3 MM) were cut to the same size as the vacuum filter support. A piece of Nylon membrane (Roche), large enough to cover the sample wells, was also cut to size before being soaked in 20x SSC buffer (Appendix). The two layers of filter paper were put into slot utensils at the top of vacuum filter support and the membrane placed on top of the filter paper. The sample well plate was inserted above the membrane and the side rails clipped with the slot utensil. A vacuum pump was connected on one side of the vacuum filter support.

0.5 to 1 μg of DNA sample was diluted with two volumes of DNA loading buffer (Appendix) and loaded evenly into the well on the sample well plate. A vacuum was applied until the solution has passed through the membrane. Then DNA on the membrane was then cross-linked as described in Section 2.3.6.2 and then subject to pre-hybridisation and hybridisation.

2.3.6.4. RNA slot blot

RNA was prepared for blotting by denaturing at 65°C for 20 min in 20 μl of total RNA (0.5-1 μg) with RNA loading buffer (Appendix). The slot blotting apparatus was prepared as described for DNA slot blots. The samples were loaded and a vacuum applied until the solution had passed through the membrane. RNA was then fixed onto the membrane by UV-crosslinking before pre-hybridisation and hybridisation was carried out.

2.3.6.5. Pre-hybridisation and hybridisation

The membrane was rolled up in a piece of nylon mesh of a size bigger than the membrane and put in a hybridisation roller bottle with 10 ml of hybridisation buffer (Appendix). After 30 min the DNA-labelled probe (25 ng/ml) was diluted to 50 μl with DNase/RNase-free H_2O , denatured at 100°C for 10 min and used for hybridisation of the blot overnight at 65°C. After hybridisation, the membrane was washed twice under low-stringency conditions with 2.0 x SSC buffer (Appendix) and twice under high-stringency conditions with 0.2 x SSC buffer (Appendix) for 20 min for each at 68°C. The membrane

was briefly washed in DIG-1 buffer (Appendix) and the blot was blocked in Blocking buffer (Appendix) for 1 h at room temperature. Anti-Digoxigenin-AP solution (Roche) was diluted 1:10 000 in blocking buffer and incubated for 30 min at room temperature. The membrane then was washed four times with DIG-1 buffer for 10 min each. Finally the membrane was rinsed for 2 min in DIG-4 buffer (Appendix).

2.3.6.6. Detection

The membrane was placed onto a piece of DNase and RNase-free hard plastic film and 1ml of the CDP-Star substrate (Sigma) was spread evenly over the surface of the nylon membrane by lowering on another piece of DNase and RNase-free film to create a liquid seal around the membrane. In a dark room, a piece of X-ray film (Kodak) was applied on top of the plastic film. The X-ray film was exposed to the membrane for 5 to 20 min in a film cassette, then immersed in developer solution (Kodak) in the dark until the bands could begin to be visualised under the red light. The film was then agitated in H₂O for 1 min to stop development and then in fixer solution (Kodak) for 2 min. Finally, the film was thoroughly rinsed with tap water.

2.3.7. Quantitative Real-Time PCR (qPCR)

2.3.7.1. Isolation of total RNA for qPCR

Total bacterial RNA for use in quantitative PCR analysis was extracted from either bacterial cultures at 0.5-0.6 OD₆₀₀ (the end of the exponential growth phase approximately 5.0×10^8 cells/ml) as detailed in Section 2.2.5, or infected human embryonic intestinal cells (INT-407 cells) treated with lysis buffer (0.1% SDS, 1% phenol pH 4.3 and 19% ethanol in water) and using an RNeasy mini kit (Qiagen) as detailed in the manufacturer's instructions. RNA extracts were stored -70°C until used.

2.3.7.2. cDNA synthesis (Reverse transcription)

For reverse transcription, cDNA Synthesis kit was used (Bioline). One μg of total RNA was mixed with reaction mixture containing 1 μl of 10 mM dNTP, 1 μl of Random Hexamer primer mix and DEPC-treated water was added to a final volume of 10 μl . The mixture was incubated at 65°C for 10 minutes and placed on ice for 2 min. The reaction mixture was mixed with a solution containing 1 μl RNase inhibitor, 0.25 μl reverse transcriptase (200 u/ μl), 4 μl 5 \times RT buffer and DEPC-treated water up to 10 μl final volume. The reaction sample was incubated at 45°C for 60 min and the reaction terminated by incubating at 70°C for 15 min. The resulting cDNA samples were stored at -80°C until required.

2.3.7.3. Optimization of qPCR

Conventional PCR was performed with each gene-primer combination to allow the optimal annealing temperature to be identified and used in the qPCR reaction containing the combined template-primer combinations. Annealing at various temperatures for each run (at 56, 57, 58, 59, 60 and 61°C) was set and the annealing temperature was finally chosen after analysis of PCR products. Furthermore, a primer concentration optimisation experiment was performed when all PCR conditions were the same apart from the primer concentration. The amplification efficiencies for all genes were performed based on the slopes of the standard curves.

2.3.7.4. Quantitative analysis of gene expression using qPCR

Relative expression of five genes (*cadF*, *ciaB*, *flaA*, *flab* and *pldA*) of all *Arcobacter* strains in this study (*A. butzleri* D2686, *A. cryaerophilus* A169/B, *A. skirrowii* 449/80, SW-OL2 and SW-DL2) was determined after 3h incubation of these strains with INT-407 cells at 37°C. Quantitative values were obtained by using the comparative threshold cycle ($\Delta\Delta C_T$) method recommended by Applied Biosystems.

The relative expression of each gene was determined three times in each of the two experimental RNA samples and was expressed as the fold difference. Positive control

was reference gene (housekeeping gene; *glnA*) and negative control (ddH₂O) was also included. Real-time RT-PCR was performed in 7900HT Fast Real-Time PCR system (Applied Biosystem) with SYBR Green Mixes (BioRad) following the manufacturer's instructions. The RT-PCR mixture was composed of 5 µl SYBR Green Mix, 1 µl of cDNA and each primer (1.4 pmol in final volume), and then Millipore water was added to a final volume of 10 µl.

The mixtures were run with following thermal cycling programme: 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15sec, annealing at 58°C (optimal T_a for all target genes) for 30 sec, and extension at 72°C for 30 sec and then 1 cycle of final extension at 72°C for 10 min. Then, the RT-PCR program was followed by a dissociation programme with a heating to 95°C for melting curve. Randomly selected samples were analysed by gel electrophoresis, which always showed PCR products of the predicted molecular weight, indicating specific amplification. To calculate relative expression, the $2^{-(\Delta\Delta CT)}$ method was used as described below:

$$\text{Fold change} = 2^{-(\Delta\Delta CT)}$$

Where $\Delta C_T = C_{T, \text{target}} - C_{T, \text{reference}}$

And $\Delta (\Delta C_T) = \Delta C_{T, \text{treated}} - \Delta C_{T, \text{untreated}}$

2.4. Statistical analysis

The majority of experiments were performed with three biological replicates. The results are presented as means \pm SD (standard deviation of the mean). All statistical analyses were performed with Minitab version (15.0) statistical software and were analysed by using Two-sample t-test. Differences were considered significant when $p > 0.05$.

CHAPTER 3 RESULTS

3.1. Isolation of *Arcobacter* species from surface water of Lothian and Fife regions

Although *Arcobacter* has been isolated from a number of different water sources across the world, it has yet to be isolated and fully studied in Scotland. This study seeks to determine the extent of *Arcobacter* contamination in Scottish surface waters and to determine whether or not these contaminants have any clinical effects compared to clinical *Arcobacter* isolates (reference type strains) isolated from symptomatic cases elsewhere.

The collection of water samples took place between April to August (2009) with 2 x 250 ml samples collected from each study water source, at eleven sampling locations (Figure 3.1). A total of eleven duplicate water samples were collected from shallow waters around the gravel shoreline of several lochs in Midlothian and Fife regions, Scotland (Table 3.1). Water samples were collected aseptically from each of the eleven study locations in sterilised glass bottles. This was achieved by immersing the container in the water source and moving it slowly away from the sampling point to minimise contamination of the sample. The bottle was then capped and chilled for transport back to the laboratory within 2-4 hrs of collection.

Table 3.1 The Sources of water samples from Lothian and Fife regions (Scotland)

No.	Sources of sample	Date of collection
1	Riccarton Loch	22.04.09
2	Duddingston Loch	19.05.09
3	Water of Leith (Currie)	14.05.09
4	Loch Ore	21.08.09
5	Loch Glow	21.08.09
6	Loch Gelay	21.08.09
7	Loch Portmore	22.08.09
9	Loch Leven	22.08.09
10	White Adder reservoir	22.08.09
11	Baddingsill reservoir	21.08.09

Bacteria were concentrated by filtration as described in Section 2.2.1. Five ml of filtrate mixture was inoculated into *Arcobacter* broth (Oxoid) supplemented with amphotericin B (8.0 mg/l), cefoperazon (4.0 mg/l) and teicoplanin (8.0 mg/l; ACT, Oxoid) and incubated microaerobically using a gas generating kit (Oxoid), at 28°C for 5 days. The

broth cultures were then streaked onto four different kinds of solid media including *Arcobacter* agar, sheep blood agar, nutrient agar, and MacConkey agar. Again, these plates were incubated aerobically and microaerobically at 28°C for 2-5 days and analysed as described below.

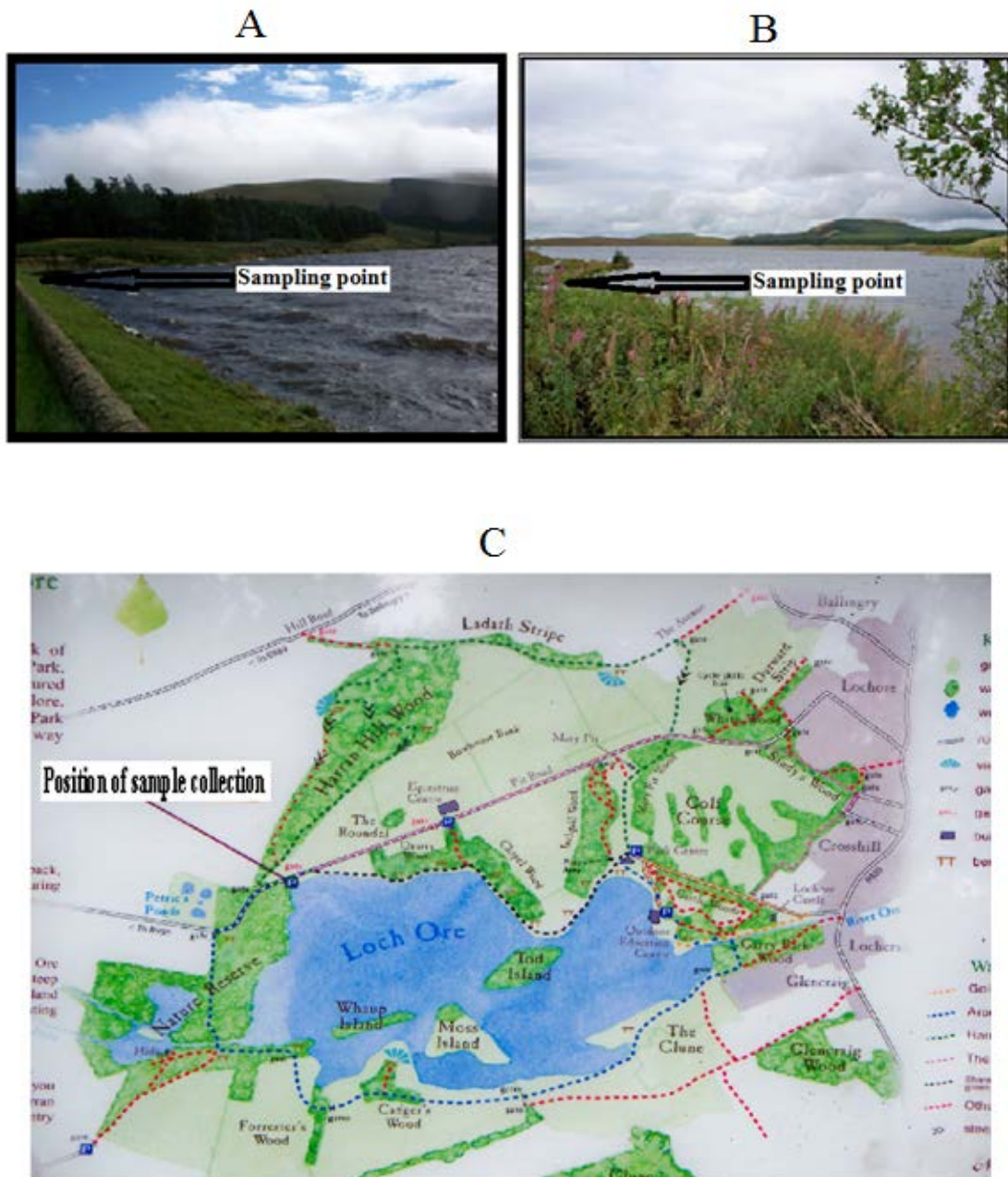


Figure 3.1 Images and map of sampling points on the loch and reservoir edges.

A: Baddingsgill reservoir; B: Loch Ore; C: map of Loch Ore.

3.1.1. Phenotypic and biochemical characteristics of water isolates

Colonies showing morphology typical of *Arcobacter* (small grey-white, round colonies) were picked from the different media (*Arcobacter* agar, sheep blood agar, nutrient agar, and MacConkey agar), purified and their identity confirmed by further phenotypic and biochemical testing.

After initial examination of colonial morphology, those showing a phenotype typical of *Arcobacter* were evaluated by Gram staining. Those isolates showing typical *Arcobacter* microscopic appearance (Gram-negative curved rods) were then tested for oxidase and catalase activities and the ability of the organisms to hydrolyse indoxyl acetate. *Arcobacter* are positive for oxidase, catalase and indoxyl acetate hydrolysis. They grow under aerobic conditions at 28°C, a trait that is used to distinguish *Arcobacter* species from *Campylobacter jejuni*.

Only six water sources (Riccarton loch, Duddingston Loch, Water of Leith, Loch Ore, Loch Portmore, and Loch Leven) of eleven sampling locations have shown results that suggest the waters might be contaminated with *Arcobacter* (Table 3.2). Genotypic characterization was performed on all the isolates that gave a positive reaction with phenotypic characteristics described above.

Interestingly, it was noticed that the suspected *Arcobacter* grew more poorly on MacConkey agar, while the growth was better on blood agar, nutrient agar or *Arcobacter* agar plates.

Table 3.2 Putative *Arcobacter* isolates identified from the sampled water sources

ISOLATE	SOURCE	MEDIUM	INCUBATION CONDITION	BIOCHEMICAL TESTS		
				Oxidase	Catalase	Indoxyl acetate hydrolysis
SW-RL1	Riccarton Loch	AA	28°C, Aerobic	++	+	+
SW-RL2		AA	28°C, Aerobic	++	+	+
SW-RL3		AA	28°C, Aerobic	++	++	+
SW-RL4		AA	28°C, Aerobic	++	+	+
SW-RL5		AA	28°C, Microaerobic	++	+	+
SW-RL6		bA	28°C, Aerobic	+	+	+
SW-RL7		bA	28°C, Microaerobic	++	++	+
SW-DL1	Duddingston Loch	NA	28°C, Aerobic	++	+	+
SW-DL2		AA	28°C, Microaerobic	++	+	++
SW-DL4		NA	28°C, Aerobic	++	++	+
SW-DL6		AA	28°C, Aerobic	++	+	+
SW-DL7		AA	28°C, Aerobic	++	+	+
SW-DL10		bA	28°C, Aerobic	++	++	++
SW-DL11		NA	28°C, Microaerobic	++	++	+
SW-DL13		NA	28°C, Microaerobic	+	++	+
SW-DL17		AA	28°C, Microaerobic	++	++	+

NA: nutrient agar; AA: Arcobacter agar; bA: blood agar; +: positive reaction; ++: strongly positive.

Table 3.2 (Continued)

ISOLATE	SOURCE	MEDIUM	INCUBATION CONDITION	BIOCHEMICAL TESTS		
				Oxidase	Catalase	Indoxyl acetate hydrolysis
SW-LC1	Water of Leith Currie	AA	28°C, Microaerobic	++	++	+
SW-LC2		AA	28°C, Aerobic	+	+	++
SW-LC6		NA	28°C, Aerobic	++	+	++
SW-LC7		bA	28°C, Aerobic	++	++	+
SW-LC8		bA	28°C, Microaerobic	+	++	+
SW-LC9	Loch Ore	bA	28°C, Aerobic	+	++	+
SW-OL1		bA	28°C, Microaerobic	+	++	+
SW-OL2		AA	28°C, Microaerobic	+	++	+
SW-PL1	Loch Portmore	NA	28°C, Microaerobic	+	++	++
SW-PL2		NA	28°C, Microaerobic	+	++	+
SW-LL1	Loch Leven	bA	28°C, Microaerobic	+	++	++

NA: nutrient agar; AA: Arcobacter agar; bA: blood agar; ++: positive reaction; +: strongly positive.

3.1.2. Species specific amplification of the 16S rDNA

The isolates giving positive reactions with the phenotypic and biochemical testing were examined by PCR assay using primers targeted to the gene encoding the 16S rRNA of *A. butzleri* D2686 and *A. skirrowii* 449/80 and the 23S rRNA gene of *A. cryaerophilus* A169/B. The selected primers (Table 2.5) amplify a 401-bp fragment from *A. butzleri*, a 257-bp fragment from *A. cryaerophilus* and a 641-bp fragment from *A. skirrowii*. The primer sets were also tested using *Bacillus subtilis* NCTC 3610 as negative control.

Using *Arcobacter butzleri* D2686, *A. cryaerophilus* A169/B and *A. skirrowii* 449/80 as positive controls, the expected amplicons were obtained using the primer combinations as described above (Figures 3.2, 3.3, 3.4 and 3.5). Eighteen of twenty-seven isolates tested exhibited an amplicon size in keeping with that of the 16S rDNA gene of *A. butzleri* (401 bp, Figure 3.2-3.4). No PCR product was generated when *Bacillus subtilis* genomic DNA was used as a template. The results are shown in Figures 3.2-3.4 and summarized in Table 3.2.

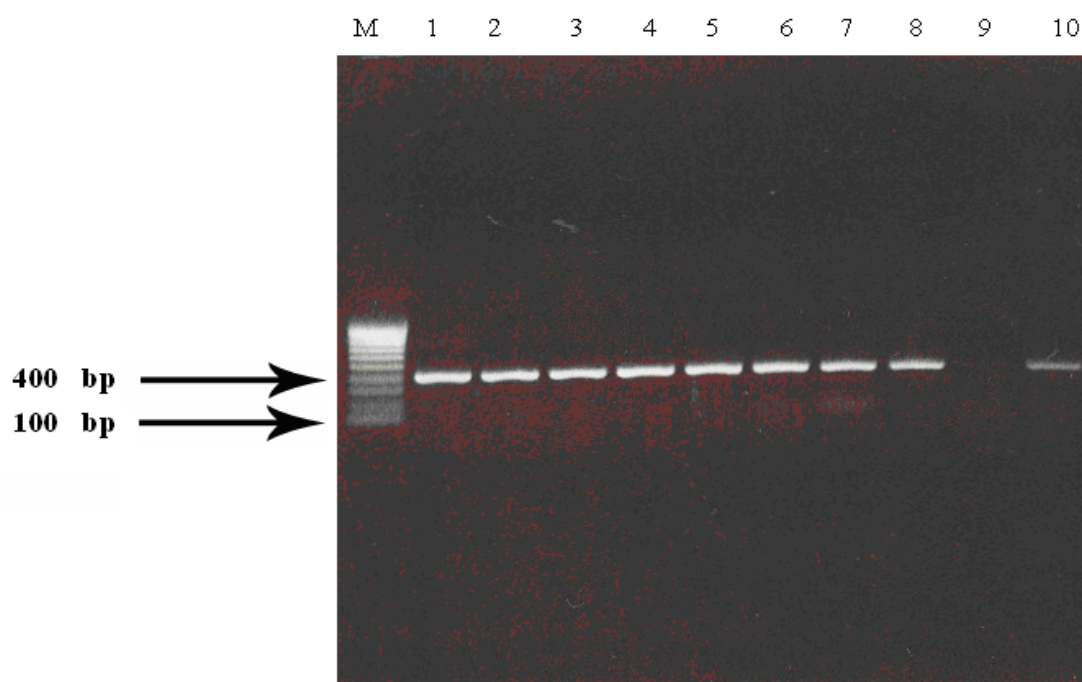


Figure 3.2 Agarose gel electrophoresis of PCR products using species-specific 16S rDNA primers (BUTZ and ARCO1) for *Arcobacter butzleri* (expected amplicon size 401-bp). M: Hyperladder IV; Lane 1: *A. butzleri* D2686; Lanes 2-8: SW-RL1-7; Lanes 9-10: SW-DL1-2.

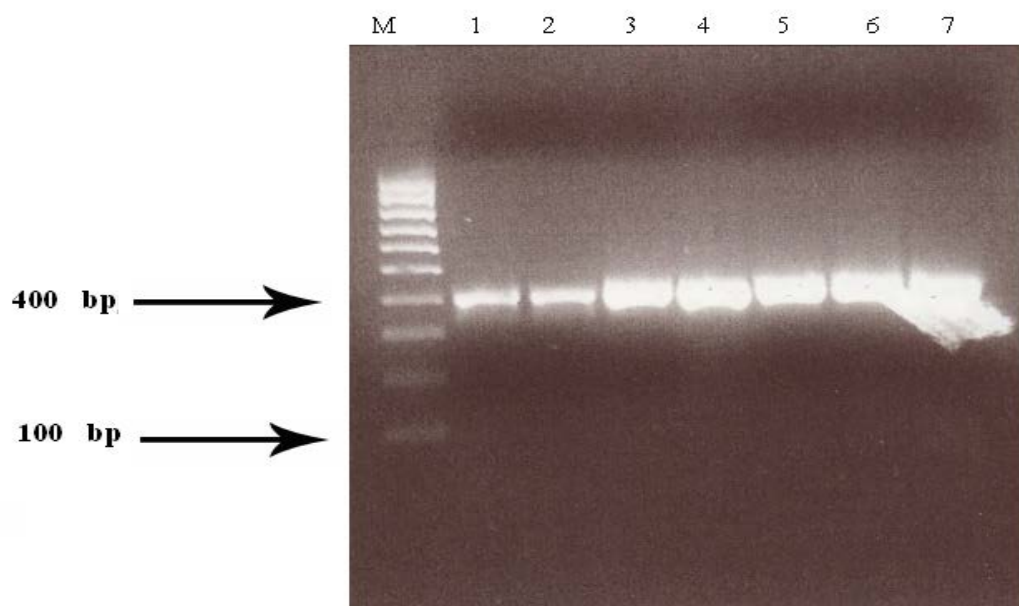


Figure 3.3 Agarose gel electrophoresis of PCR products with using species-specific primers (BUTZ and ARCO1) for the 16S rDNA gene of *Arcobacter butzleri* (401-bp). M: Hyperladder IV; Lane 1: *A. butzleri* D2686; Lane 2: SW-DL11; Lane 3: SW-LC1; Lanes 4-5: SW-LC8-9; Lane 6: SW-LL1; Lane 7: SW-RL1.

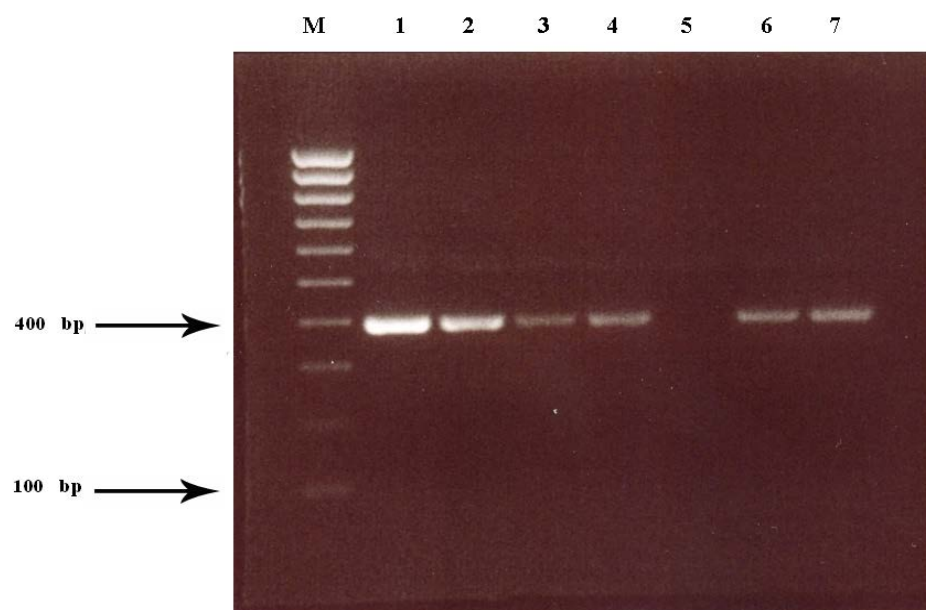


Figure 3.4 Agarose gel electrophoresis of PCR products with using species-specific primers (BUTZ and ARCO1) for the 16S rDNA gene of *Arcobacter butzleri* (401-bp). M: Hyperladder IV; Lane 1: *A. butzleri* D2686; Lane 2: SW-RL2; Lane 3: SW-PL1; Lane 4: SW-PL2; Lane 5: *Bacillus subtilis* NCTC 3610; Lane 6: SW-OL1; Lane 7: SW-OL2.

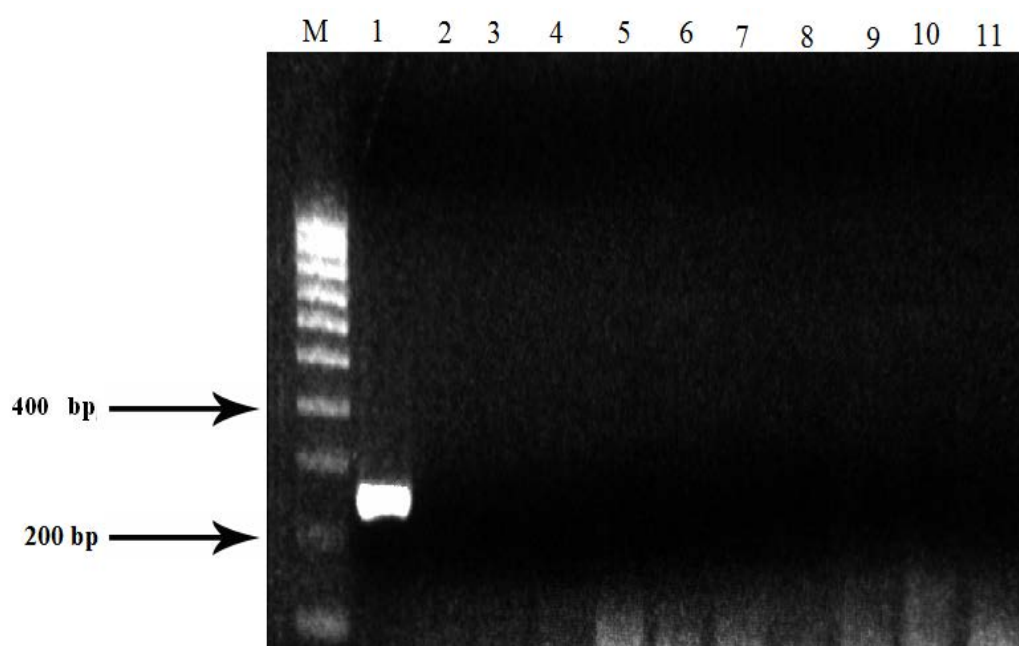


Figure 3.5 Agarose gel electrophoresis of PCR products using species-specific 23S rDNA primers (CRY1 and CRY2) for *Arcobacter cryaerophilus* (expected amplicon size 257-bp). M: Hyperladder IV; Lane 1: *A. cryaerophilus* A169/B; Lane 2: *Bacillus subtilis* NCTC 3610; Lanes 3-11: SW-DL1, 2, 4, 6, 7, 10, 13, 17.

The putative *Arcobacter* isolates were also examined by PCR assay using primers CRY1 & CRY2 and SKIR & ARCO2, targeted to the genes encoding the 23S rDNA gene of *A. cryaerophilus* and the 16S rDNA gene of *A. skirrowii*, respectively. No amplification was seen in the PCR reactions when the isolates were tested with these primers, however, positive results were seen when *A. cryaerophilus* A169/B (Figure 3.5) and *A. skirrowii* 449/80 were introduced as positive controls. This confirms that the primers do work and appear to be strain specific.

Table 3.3 PCR of the putative *Arcobacter* water isolates using species-specific primers.

Strain and water isolates	<i>A. butzleri</i>	<i>A. cryaerophilus</i>	<i>A. skirrowii</i>
<i>A. butzleri</i> D2686	+	-	-
<i>A. cryaerophilus</i> A169/B	-	+	-
<i>A. skirrowii</i> 449/80	-	-	+
<i>B. subtilis</i> NCTC 3610	-	-	-
SW-RL1	+	-	-
SW-RL2	+	-	-
SW-RL3	+	-	-
SW-RL4	+	-	-
SW-RL5	+	-	-
SW-RL6	+	-	-
SW-RL7	+	-	-
SW-DL1	-	-	-
SW-DL2	+	-	-
SW-DL4	-	-	-
SW-DL6	-	-	-
SW-DL7	-	-	-
SW-DL10	-	-	-
SW-DL11	+	-	-
SW-DL13	-	-	-
SW-DL17	+	-	-
SW-LC1	+	-	-
SW-LC2	-	-	-
SW-LC6	-	-	-
SW-LC7	-	-	-
SW-LC8	+	-	-
SW-LC9	+	-	-
SW-OL1	+	-	-
SW-OL2	+	-	-
SW-PL1	+	-	-
SW-PL2	+	-	-
SW-LL1	+	-	-

- : negative reaction; +: positive reaction.

3.1.3. Genomic fingerprinting

RAPD-PCR was used to assess the intraspecific diversity of all putative *Arcobacter* isolates and discriminate them based on genetic diversity data from combined numerical analysis of the patterns obtained from this technique.

All eighteen of the putative *Arcobacter* isolates which gave PCR products specific for the 16S rDNA gene of *Arcobacter butzleri* were assessed using RAPD-PCR. RAPD-PCR profiles, which were obtained by using the OPB17 primer, allowed comparison of the genetic relationships between these putative *Arcobacter* isolates. The RAPD-PCR fingerprinting profiles of the isolates were compared for similarity by visual inspection of the band profiles and by using Labimage software (BIORAD). Sterile millipure water was used as negative control in this study.

The results shown in Figures 3.6 and 3.7 demonstrate the variety of RAPD-PCR profiles obtained from specimens isolated from different water sources (e.g. lochs and reservoir); however, there was high similarity of RAPD-PCR profiles when specimens from the same water source were compared. The number of visible amplified fragments ranged from 2 to 12 and included bands of between 100-2500 bp in size that were shared by each of the eighteen putative *Arcobacter* isolates (Figures 3.6, 3.7). For example, the RAPD-PCR profiles obtained from specimens SW-RL1 and SW-RL7 displayed high similarity and they were both isolated from Riccarton loch. In contrast, there were more significant differences between RAPD-PCR profiles of the isolates obtained from the Water of Leith and Currie (SW-LC1, SW-LC8 and SW-LC9).

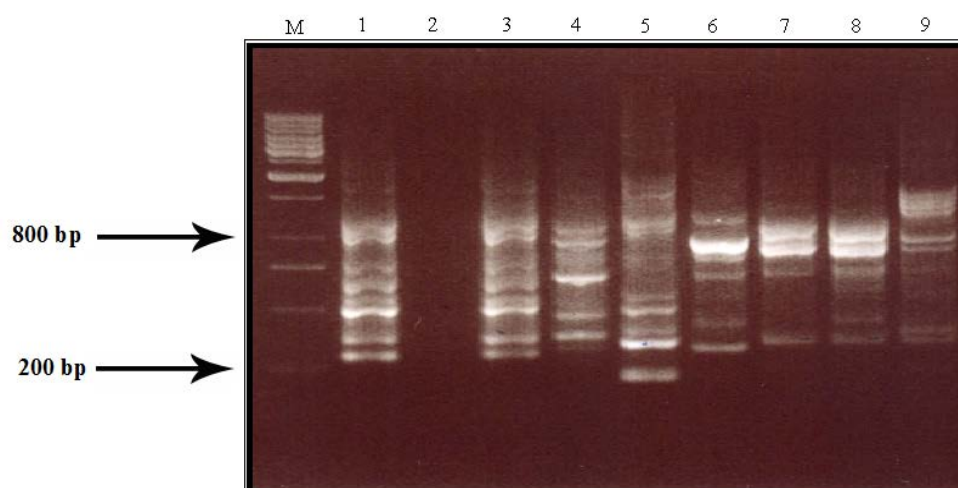


Figure 3.6 RAPD-PCR profiles of putative *Arcobacter* isolates using the OPB17 primer. M: Hyperladder I; Lane 1: SW-RL1; Lane 2: negative control; Lane 3: SW-RL7; Lane 4: SW-DL2; Lane 5: SW-OL1; Lane 6: SW-LC1; Lane 7: SW-LC8; Lane 8: SW-LC9; Lane 9: SW-PL1.

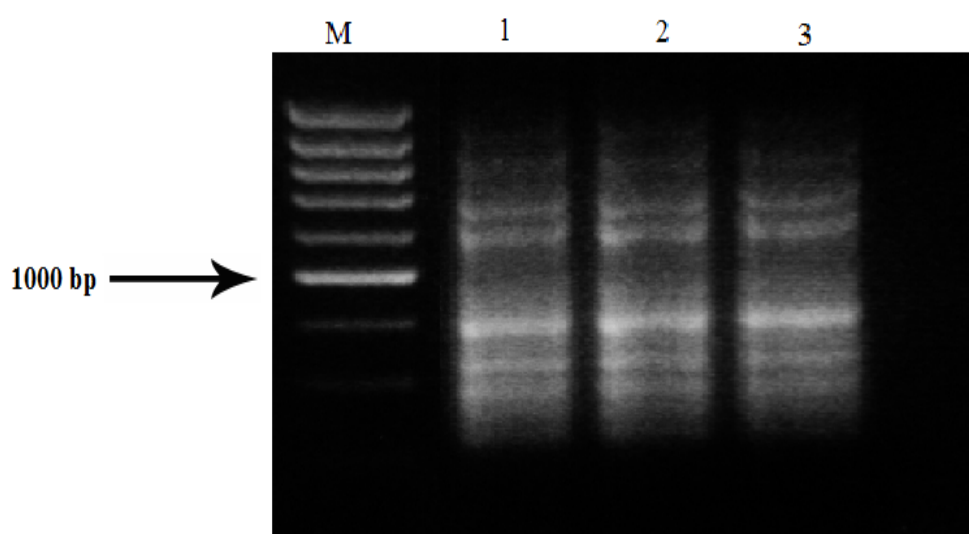


Figure 3.7 RAPD-PCR profiles of putative *Arcobacter* isolates using OPB17 primer. M: Hyperladder I; Lane 1: SW-DL2; Lane 2: SW-DL11; Lane 3: SW-DL17.

RAPD-PCR profiles obtained with putative *Arcobacter* isolates from Duddingston loch (SW-DL2, SW-DL11 and SW-DL17) could not be distinguished from each other. These three strains generated similar profiles with a small number of bands, some which were of low intensity (Figure 3.7).

3.1.4. Analysis of the 16S rRNA sequences of water isolates

To confirm bacterial phylogeny and taxonomy of the putative *Arcobacter* isolates, 16S rRNA sequencing was used. Water isolates producing a PCR amplification product of a size in keeping with that of the *Arcobacter butzleri* type strain were subjected to 16S rDNA gene sequence analysis. Partial sequence analysis showed that each of the 16S rDNA sequences tested were from Gram-negative bacteria, although the majority of sequences provided ambiguous identifications in most instances. Only two water isolates sequenced (SW-DL2 and SW-OL2) were confirmed as belonging to the *Arcobacter* cluster by this method (Figure 3.8, 3.9).

The 16S rDNA gene sequence of SW-DL2 showed identity values of between 95 and 97 % with the sequences of the *Arcobacter* genus and the highest value (97%) was obtained with *Arcobacter butzleri* strain ED-1. The nucleotide sequence of the 16S rDNA gene in SW-OL2 shares 92% identity over 1096 bases with that of *Arcobacter butzleri* strain RM4018 and the 16S rDNA gene of SW-OL2 falls firmly within the genus *Arcobacter* (91-93% identity). Therefore, phylogenetic analysis based on 16S rDNA gene sequences revealed that two of the putative *Arcobacter* isolates (SW-DL2 and SW-OL2) fall within the *Arcobacter* cluster (Figure 3.8, 3.9). The level of 16S rDNA gene sequence identity between SW-DL2 and SW-OL2 is 92%.

The other putative *Arcobacter* isolates did not show any similarity with the *Arcobacter* cluster and were misidentified at the genus level. They did, however, show high bootstrap values with uncultured bacteria and other *Proteobacteria*, such as; *Pseudomonas brenneri*, *Pseudomonas alcaligenes*, *Microvirgula aerodenitrificans*, *Vogesella indigofera* and *Laribacter hongkongensis*.

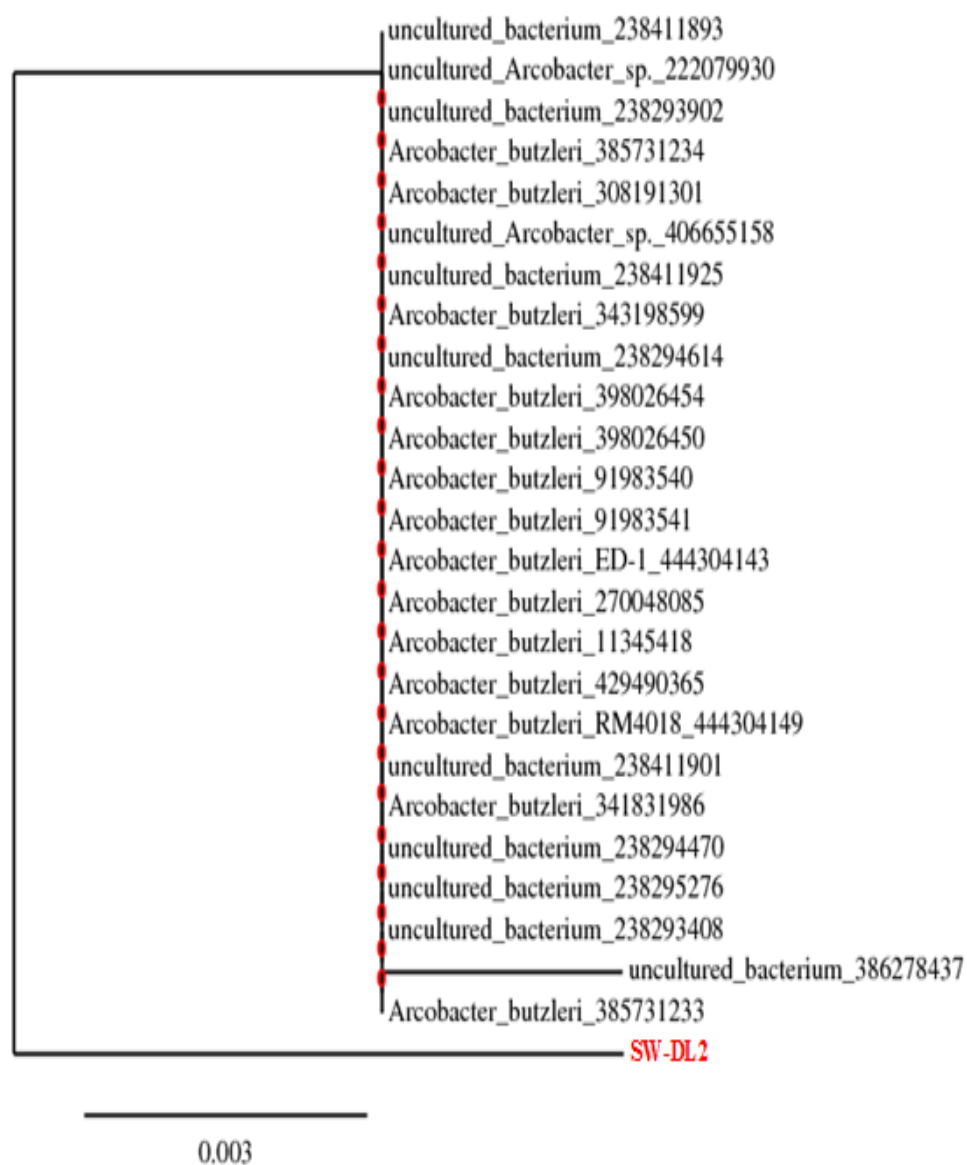


Figure 3.8 Phylogenetic tree based on 16S rDNA gene sequences showing the phylogenetic position of SW-DL2 within the *Arcobacter* cluster (>96% identity).

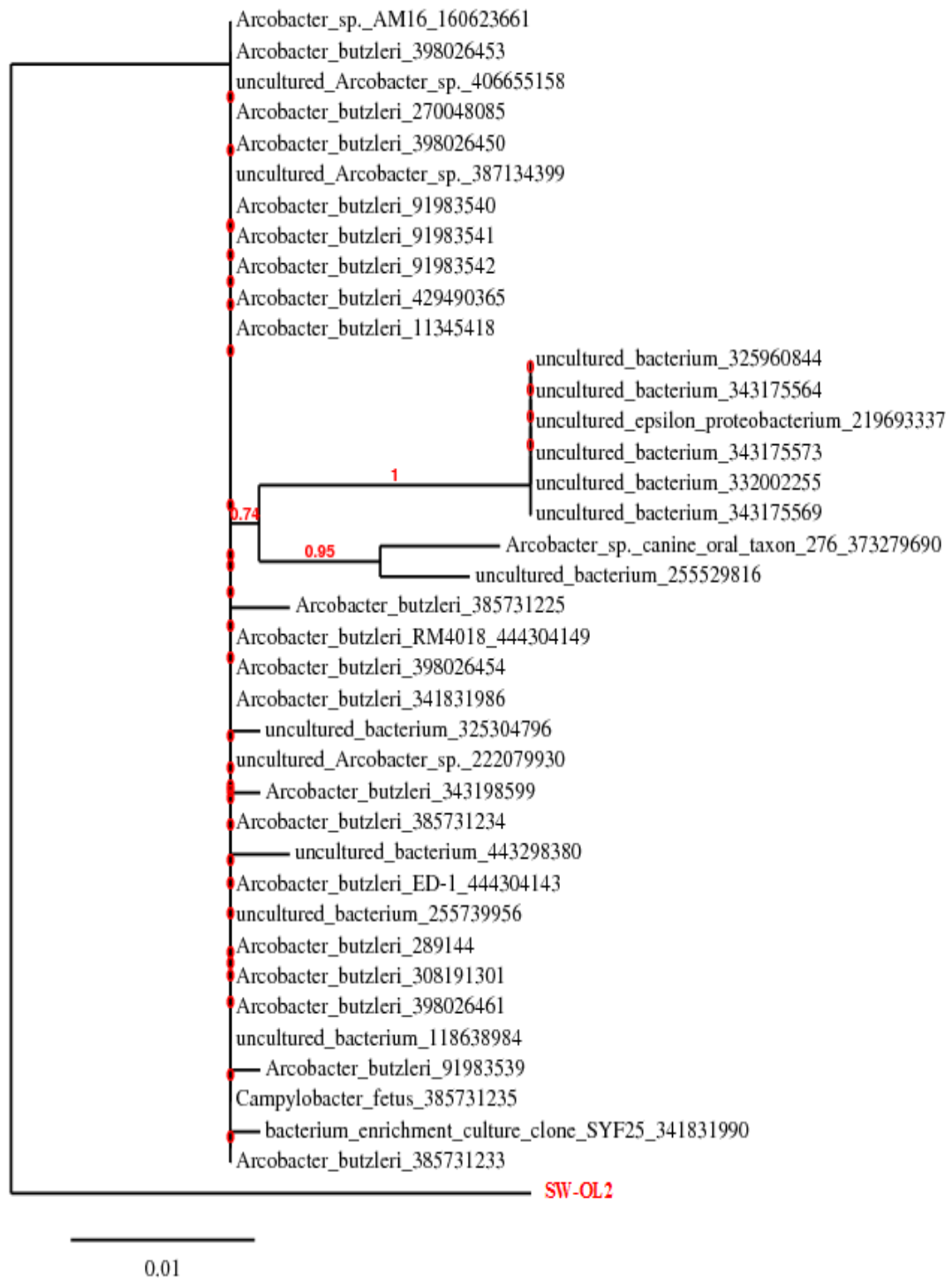


Figure 3.9 Phylogenetic tree based on 16S rDNA gene sequences showing the phylogenetic relationship of SW-OL2 with the genus *Arcobacter* (>90% identity).

The data presented in this section have shown that only two out of eleven surface water sources were clearly contaminated with *Arcobacter* species (18%) and that these two *Arcobacter* isolates, were obtained from Duddingston Loch and Loch Ore (SW-DL2 and SW-OL2 respectively). This study demonstrates that the sequencing of 16S rDNA is a suitable technique to identify bacteria at the genus level. However, traditional phenotypic tests have their uses in narrowing down the number of isolates from environmental samples.

Having successfully identified two of the water isolates as *Arcobacter* species, as a next step it was decided to compare the isolates with known type strains of *Arcobacter* based on their enzyme activities and pathogenicity determinants.

3.2. Qualitative analysis of enzyme activity of *Arcobacter* strains

To investigate the lipolytic activity of all *Arcobacter* strains used in this study, their ability to degrade lipid was examined by inoculation of bacterial culture ($4\text{--}5.0 \times 10^8$ CFU/ml) onto TSA supplemented with 1% egg yolk emulsion and 1% Tween 80 (Section 2.3.1.4). Elastase, caseinase, gelatinase and haemolytic activities were also investigated using plate assays for these *Arcobacter* strains.

All reference strains (*Arcobacter butzleri* D2686, *Arcobacter cryaerophilus* A169/B, and *Arcobacter skirrowii* 449/80) and the two water *Arcobacter* isolates (SW-DL2 and SW-OL2) degraded elastin and gelatin; none produced phospholipase, lipase or caseinase. Neither the reference strains nor the water isolates showed haemolysis on blood agar plates made with blood from a variety of sources including human, sheep, chicken, cattle, and horse (Table 3.4).

Table 3.4 Enzyme activities of references strains and water *Arcobacter* isolates

Strain	Phospholipase activity (egg yolk)	Lipase activity (Tween 80)	Protease activity			Haemolysis of erythrocytes from				
			Casein	Gelatin	Elastin	Sheep	Horse	Chicken	Cattle	Human
<i>A. butzleri</i> D2686	-	-	-	+	++	-	-	-	-	-
<i>A. cryaerophilus</i> A169/B	-	-	-	+	++	-	-	-	-	-
<i>A. skirrowii</i> 449/80	-	-	-	+	++	-	-	-	-	-
SW-DL2	-	-	-	+	++	-	-	-	-	-
SW-OL2	-	-	-	+	++	-	-	-	-	-

(-) negative ; (+) diameter of zone of opalescence of 3 mm; (++) diameter of zone of opalescence of 3.5-6 mm.

3.3. Quantitative characterisation of the Extracellular Proteins (ECPs)

Arcobacter species, especially pathogenic ones causing disease in humans or animals, are likely to have a range of extracellular products associated with pathogenicity and environmental adaptability. In this series of experiments, the extracellular proteins that may mediate pathogenicity, including secreted enzymes such as caseinase, gelatinase, lipase, phospholipase and putative haemolysins, were further characterized by assaying the enzyme activities of the purified ECP extracts.

Extracellular protein extracts were prepared and the total protein concentration determined using the Bradford assay. All strains appeared to produce similar levels of extra-cellular protein, in the range 1099-1318 µg/ml, from 40 ml of bacterial culture grown in *Arcobacter* broth for 5 days. The extracellular protein extracts were then subjected to further quantitative analysis. Phospholipase and lipase activity could not be detected in any of ECP preparations, while caseinase and gelatinase activities were shown to be associated with all ECP preparations (Table 3.5). The ECPs of SW-DL2 and SW-OL2 possessed high gelatinase activity (310 and 224 units/ml, respectively) compared with *A. butzleri* type strain D2686 and also had the highest caseinase activity of all the strains tested in this study (Table 3.5). The haemolytic assay was performed on blood from human and various animal species (sheep, chicken, cattle, horse) and the results showed that all of the strains from which ECP was prepared were able to lyse the erythrocytes of chicken and sheep (Figure 3.11), to varying degrees (Table 3.5). In addition, one strain (*A. butzleri* D2686) was also able to haemolyse human erythrocytes (Figure 3.10). None of the ECPs from the strains tested were able to lyse erythrocytes from either cattle or horses (Table 3.5).

It should be emphasised from sections 3.2 and 3.3 that of the *Arcobacter* strains in this study none produced phospholipase and lipase, while all degraded elastin which causes proteolytic damage to connective tissue and contributes to their pathogenicity. The role of hemolysins in the virulence of *Arcobacter* species to animals and humans was not well documented although interestingly most *Arcobacter* strains in this study were found to be poor producers of hemolysins. The ECP of all strains caused haemolysis of two species of erythrocytes (sheep and chicken) and *A. butzleri* strain D2686 showed also a poor haemolysis of human erythrocytes.

Table 3.5 Characteristics of extracellular products (ECP) of *Arcobacter* strains

Strain	Protein content (µg/ml)	Phospholipase activity (egg yolk)	Lipase activity (Tween 80)	Units of caseinase activity	Units of gelatinase activity	Haemolytic activity against different erythrocytes				
						Horse	Sheep	Cattle	Chicken	Human
<i>A. butzleri</i> D2686	1161	-	-	4	51	-	++	-	+	++
<i>A. cryaerophilus</i> A169/B	1318	-	-	3	91	-	++	-	+++	-
<i>A. skirrowii</i> 449/80	1189	-	-	4	50	-	++	-	+++	-
SW-OL2	1151	-	-	11	224	-	++	-	+	-
SW-DL2	1099	-	-	38	310	-	++	-	++	-

(-) absence of degradative or haemolytic activity; (+) weak activity, with zone of opalescence of ≥ 3 mm; (++) easily apparent activity, with zone of opalescence of 3.5-6 mm; and (+++) strong activity, with zone of opalescence ≥ 6.5 -9 mm

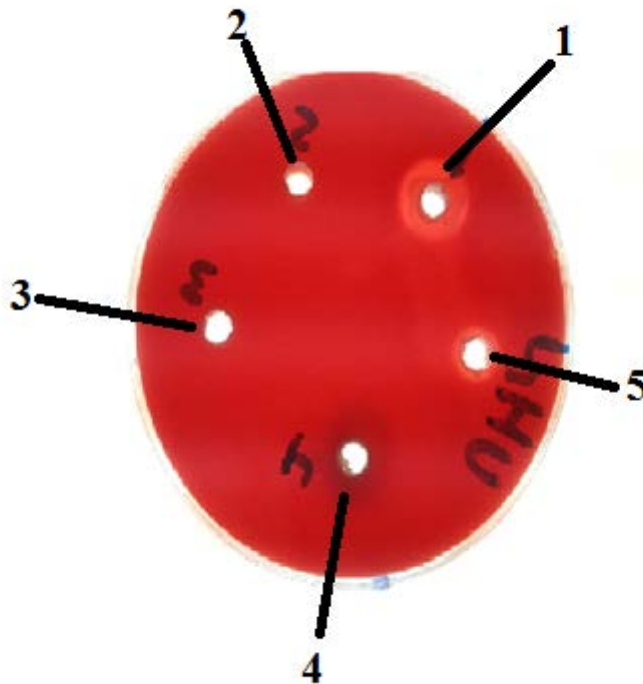


Figure 3.10 The haemolytic activity of purified ECPs against human blood. 1: *A. butzleri* D2686; 2: *A. cryaerophilus* A169/B; 3: *A. skirrowii* 449/80; 4: SW-DL2; 5: SW-OL2.

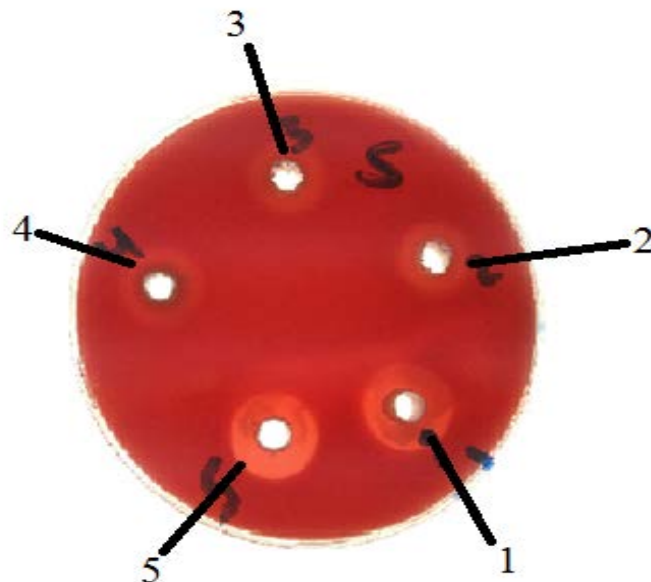


Figure 3.11 The haemolytic activity of purified ECPs against sheep blood. 1: *A. butzleri* D2686; 2: *A. cryaerophilus* A169/B; 3: *A. skirrowii* 449/80; 4: SW-DL2; 5: SW-OL2.

3.4. SDS-PAGE analysis of subcellular fractions of *Arcobacter*

SDS-PAGE was used to separate complex mixture of proteins comprised of subcellular components and allowed comparison between *Arcobacter* strains in this study. Whole cell lysates were prepared as described in Section 2.3.2 and the subcellular components were analysed by SDS-PAGE on a 12% polyacrylamide gel. A comparative analysis of putative virulence protein profiles of these strains was then carried out.

3.4.1. Whole cell protein extracts

The whole cell protein profiles across the type strains and water isolates were slightly similar in appearance. More than 20 protein bands could be resolved, ranging in size from 230 kDa to less than 30 kDa, as determined by visual assessment of their approximate molecular masses (Figure 3.12). Major protein bands around 40 and 80 kDa with a triplet of bands just below 100 kDa, were apparent in some strains.

Although the electrophoretic banding patterns are insufficient for accurate discrimination between the strains, some differences were noticeable. For example, the relative intensities of the *Arcobacter butzleri* D2686 bands were fairly consistent and *A. cryaerophilus* A169/B and *A. skirrowii* 449/80 had the same arrangement of major bands but with a relatively greater intensity band at 40 kDa. Water specimens SW-DL2 and SW-OL2 had a banding pattern most similar to that of *A. butzleri* D2686 (Figure 3.12).

3.4.2. Outer membrane proteins

Outer membrane proteins were prepared from plate cultures by ultrasonic treatment followed by ultracentrifugation and resolubilization of the membrane proteins. The preparations were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie blue before visual analysis of outer membrane expression was made (Figure 3.13).

The outer membrane proteins profiles were more varied than for the whole cell protein profiles. All strains had more than 10 bands, with varied representative bands ranging in size between 30 and 200 kDa (Figure 3.13). Closer inspection of the protein profiles revealed that *Arcobacter* isolates SW-DL2 and SW-OL2 had a similar banding pattern to each other and to *Arcobacter butzleri*, with two major bands in common at ~97 and ~34 kDa. *Arcobacter butzleri* had an additional strong band of less than 30 kDa, not seen in either of the water isolates. This contrasted with the other reference strains (*A. cryaerophilus* and *A. skirrowii*), which had multiple distinct bands and different banding profiles (Figure 3.13). The major outer membrane proteins for these strains appeared at 34.6, 42.7, 42.7, 97.2 and 98.2 kDa (*A. butzleri* D2686, *A. cryaerophilus* A169/B, *A. skirrowii* 449/80, SW-DL2 and SW-OL2 respectively).

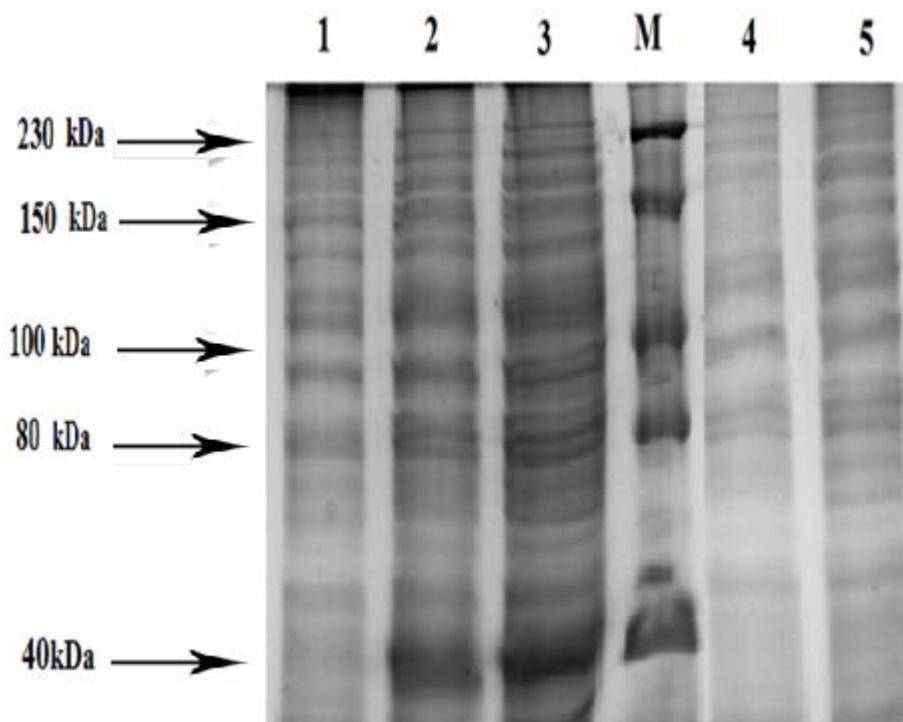


Figure 3.12 Whole cell protein SDS-PAGE profiles of *Arcobacter* strains and water isolates (each sample was loaded in 20 μ l of protein suspension). M: protein marker; 1: *A. butzleri* D2686; 2: *A. cryaerophilus* A169/B; 3: *A. skirrowii* 449/80; 4: SW-DL2; 5: SW-OL2.

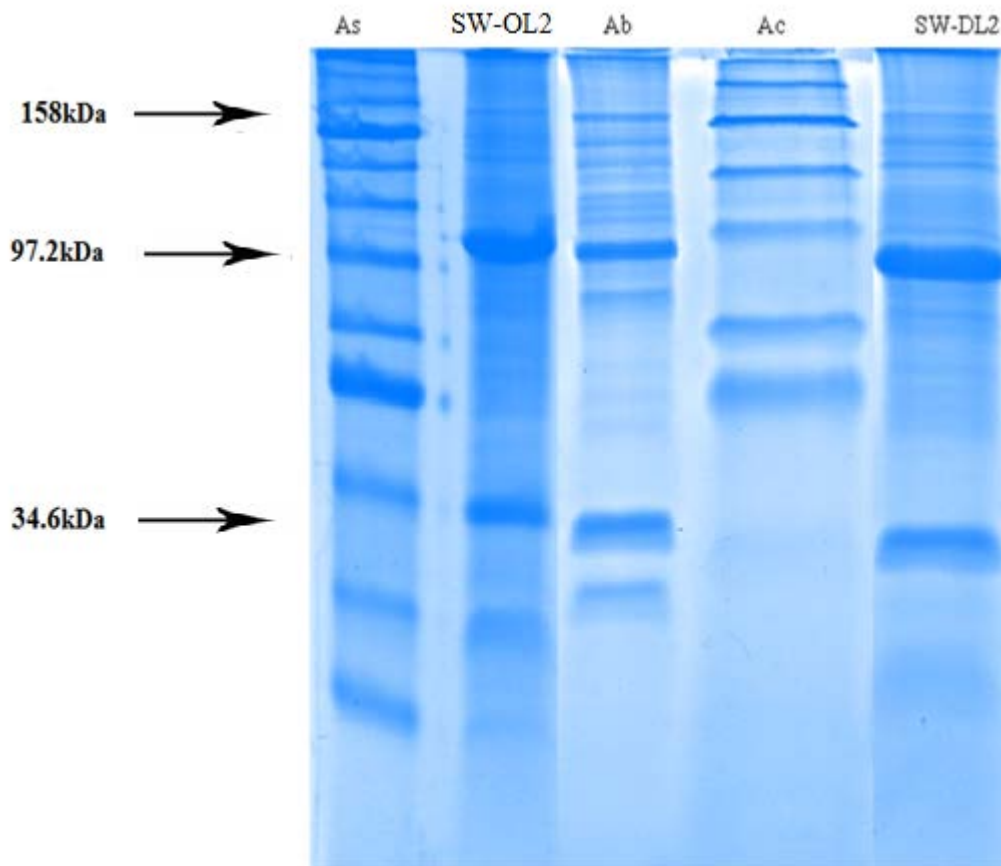


Figure 3.13 Outer membrane protein SDS-PAGE profiles of *Arcobacter* strains (each sample was loaded in 20 μ l of protein suspension). As: *A. skirrowii* 449/80; SW-OL2; Ab: *A. butzleri* D2686; Ac: *A. cryaerophilus* A169/80; SW-DL2.

3.4.3. ECP

Generally, the ECP profiles showed a very high level of similarity between *A. butzleri* and the two water isolates (SW-DL2 and SW-OL2), and between *A. cryaerophilus* A169/B and *A. skirrowii* 449/80. The ECPs of all strains had bands of molecular weight between 8-160 kDa and most strains shared prominent protein bands at 80, 70 and 25kDa (Figure 3.14).

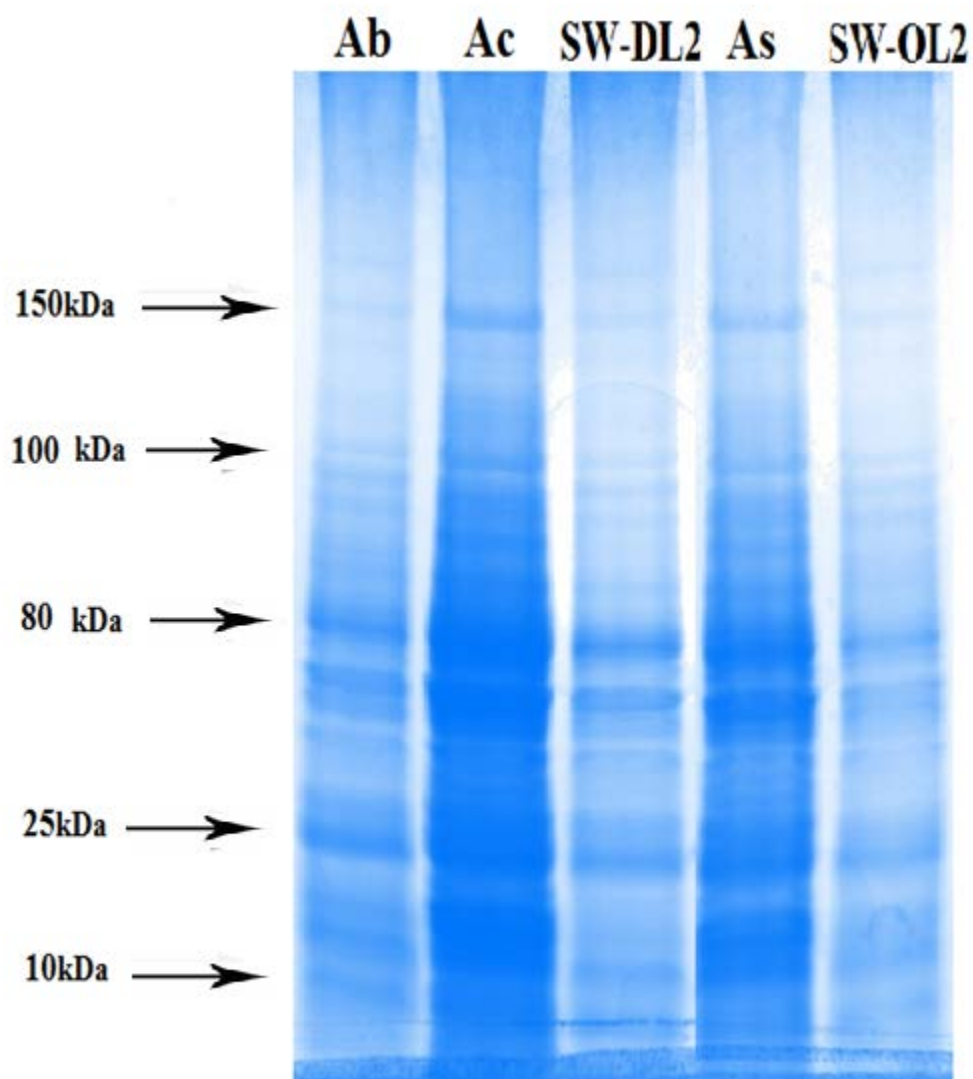


Figure 3.14 Extracellular protein SDS-PAGE profiles of *Arcobacter* strains (each sample was loaded in 20 μ l of protein suspension). Ab: *A. butzleri* D2686; Ac: *A. cryaerophilus* A169/80; SW-DL2; As: *A. skirrowii* 449/80; SW-OL2

SDS-PAGE analysis of whole cell proteins, outer membrane proteins, and ECP products demonstrated that only the outer membrane proteins showed significant differences between the reference strains (*Arcobacter butzleri* D2686, *Arcobacter cryaerophilus* A169/B, and *Arcobacter skirrowii* 449/80) and the water derived isolates (SW-DL2 and SW-OL2).

3.5. Molecular cloning of the *cadF* gene and characterisation of *cadF* mutants

The mechanisms of *Arcobacter* invasion/colonisation are poorly understood but that initial attachment of pathogenic bacteria to host cells is commonly associated with pathogenesis – particularly with those pathogens that cause enteric disease. Initial recognition and attachment is often mediated through the expression of proteins on the bacterial cell surface that recognise and attach to molecules associated with the host. Most bacterial adhesins recognise and bind to the host extracellular matrix (ECM) via proteins such as fibronectin (Fn), fibrinogen/fibrin and collagen. One of the most common extracellular matrix molecules that binds to these adhesins, is fibronectin (Fn), adhesins of which have been identified in many different pathogenic bacteria (Section 1.6.2). To investigate the potential role of the *Arcobacter* CadF protein as an adhesin for host cells interaction, *cadF* mutants were generated in *A. butzleri* D2686 and SW-OL2 as described in the methods chapter (Section 2.3.4).

3.5.1. Cloning and sequencing of the *cadF* gene

The *cadF* genes were amplified (Figure 3.15) from two strains, *A. butzleri* D2686 and the water isolate (SW-OL2) using conventional PCR with primers which were designed from the genome of *Arcobacter butzleri* strain RM4018, as described in previous Section 2.2.12.1 and in Table 2.5 (*CadF*-FP1 and *CadF*-RP1 primers for wild type strain and *CadF3* Fwd and *CadF3* Rev for the water isolate). The pCR2.1-TOPO vector (Invitrogen) is a plasmid with a size of 3.9 kb and the PCR products of the putative *cadF* gene from both strains were ligated and cloned at the multiple cloning sites (MCS) of this vector (Figure 3.16). These constructed plasmids with a cloned *cadF* gene were transformed into the chemically competent *E. coli* strain XL1-Blue MRF and the clones were subsequently screened for white colonies which contained plasmid with the putative *cadF* gene. The colony suspension was used as template for PCR with appropriate primers (M13 forward and reverse) to examine whether the putative *cadF* gene had been cloned into the pCR2.1-TOPO vector (Figure 3.17). Restriction endonuclease digestion was also carried out to detect if the *cadF* was cloned into the pCR2.1-TOPO vector (Figure 3.18). Sequencing of target DNA fragments was also

used to further identify the putative *cadF* genes using appropriate primers as detailed in Table 2.5. The *cadF* gene sequence of SW-OL2 and *A. butzleri* D2686 showed high level of sequence identity (96% and 100% respectively) with a putative gene that encodes fibronectin-binding protein (Fbp) in *A. butzleri* RM4018 (Figure 3.19).

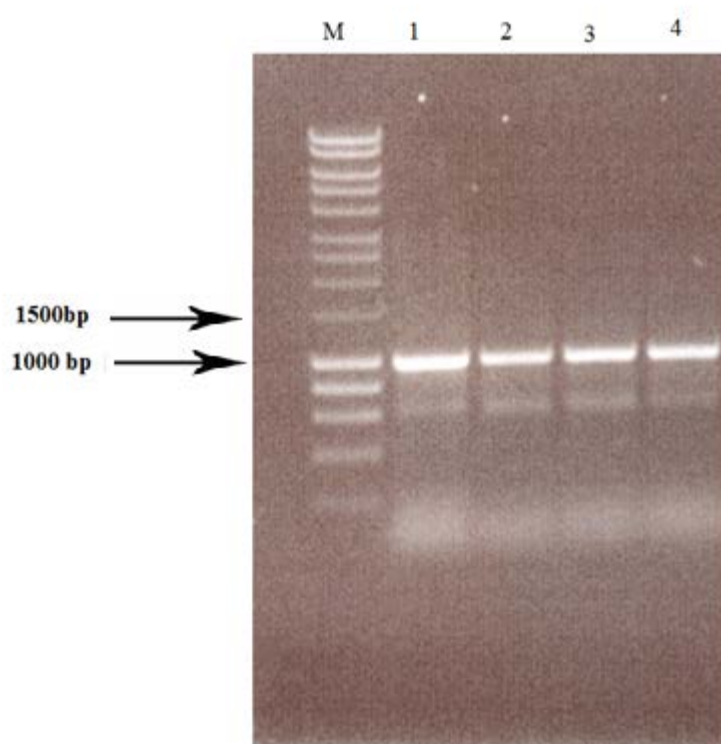


Figure 3.15 Amplification of *cadF* genes. Lanes 1-2: *A. butzleri* D2686; Lanes 3-4: SW-OL2; M: Hyperladder I DNA marker.

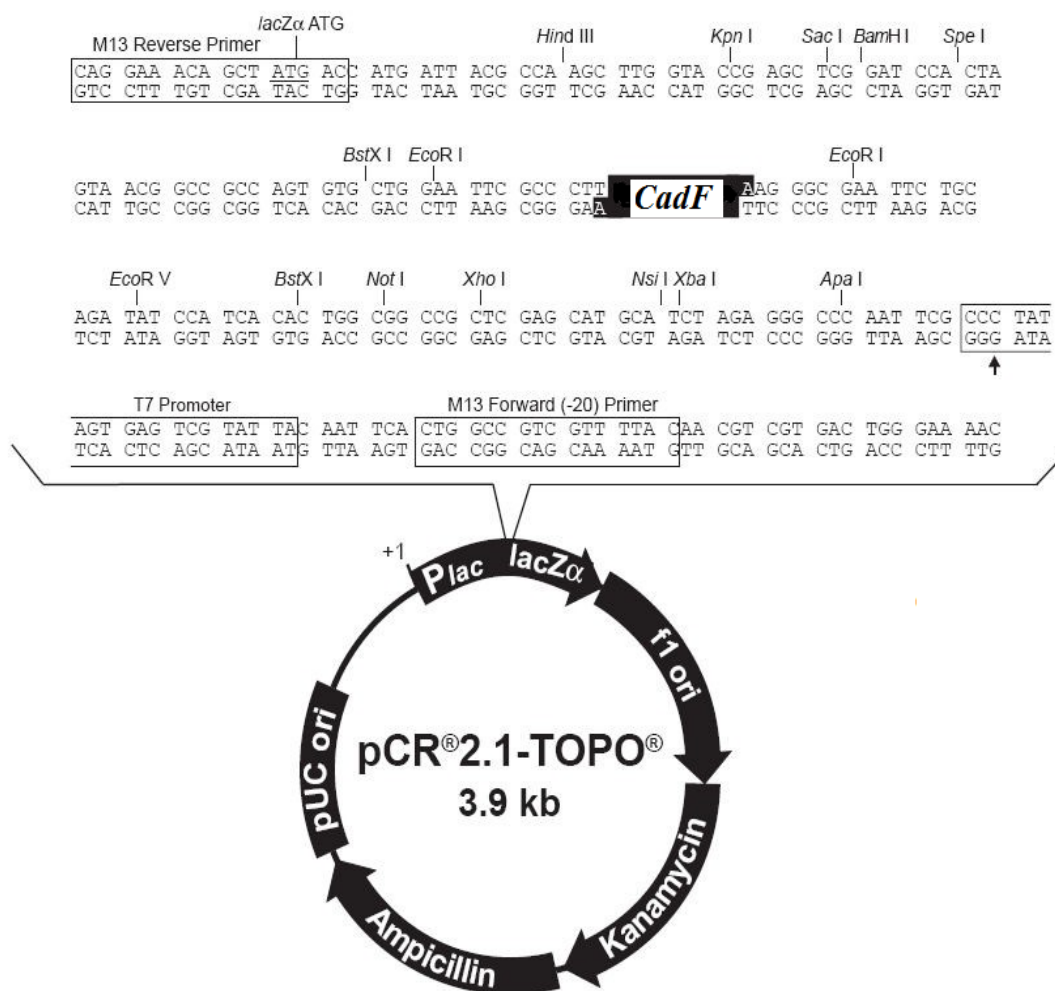


Figure 3.16 Restriction map of pCR2.1-TOPO vector showing where the PCR product of putative *cadF* gene was ligated and cloned at the multiple cloning sites (MCS) of this vector.

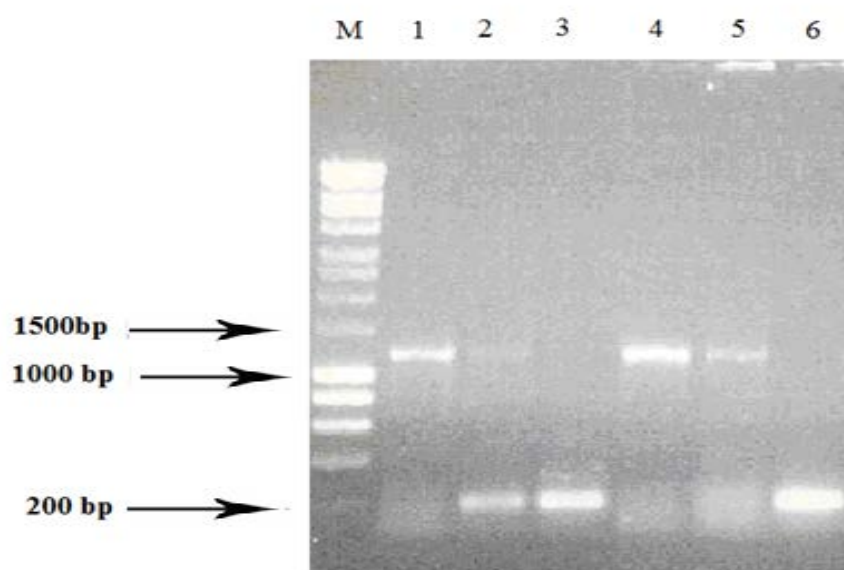


Figure 3.17 PCR screening using M13 forward and reverse primers to check the transformation. M: Hyperladder I; Lane 1: white colony with the *cadF* gene of *A. butzleri* D2686 in pPCR-TOPO plasmid; Lanes 2-3: blue colonies without the *cadF* gene; Lanes 4-5: white colonies with the *cadF* gene of SW-OL2 in pPCR-TOPO plasmid; Lane 6: blue colony without the *cadF* gene.

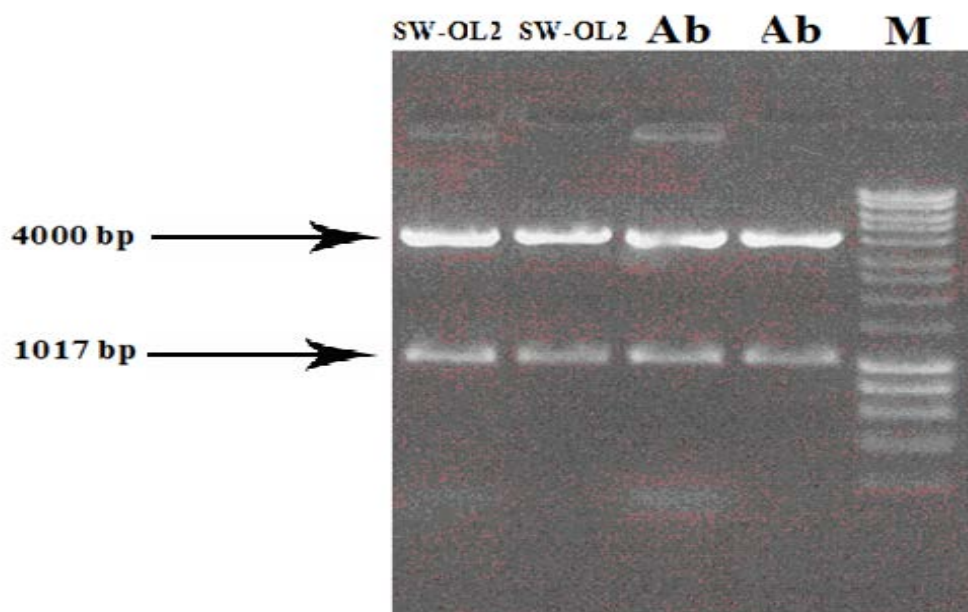


Figure 3.18 *EcoRI* digestion of pPCR-TOPO plasmids containing the cloned *cadF* gene. 4 kb band represents the TOPO vector and the 1017 bp band represents the *cadF* gene; M: hyper ladder I; Ab: *A. butzleri* D2686.

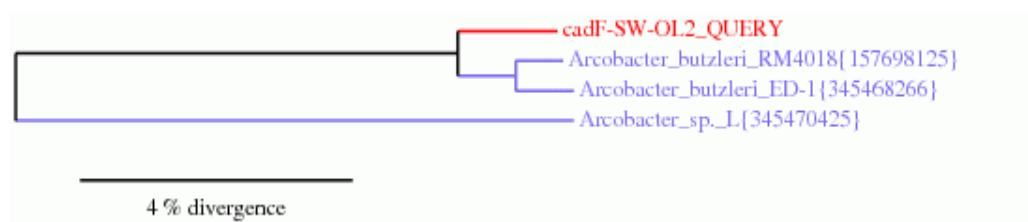


Figure 3.19 Phylogenetic tree of *cadF* gene sequences, showing the phylogenetic position of *cadF* gene of SW-OL2 within the *cadF* *Arcobacter* cluster.

3.5.2. Generation of a *cadF* mutant

The *cadF* gene and its flanking regions from *A. butzierei* D2686 and water isolate (SW-OL2) were amplified by PCR using the primers *CadF3* Fwd: 5'- CAA TAA TAG GAG AAG CAA TT-3' and *CadF3* Rev: 5'- TGT TAC AGT TAA AAT GTC AA -3'. The fragment was then ligated into the pCR-2.1 TOPO-cloning vector (Invitrogen) and sequenced to confirm that the whole *cadF* gene had been amplified and no PCR-generated errors had been introduced. The flanking regions of the cloned *cadF* gene were then digested using *EcoRI*, and the *EcoRI* fragment carrying the *cadF* gene was cloned into *EcoRI* digested pBluescript KS(+) and transformed into *E. coli* XL1-Blue MRF (Figure 3.20). An inactivated *cadF* gene in strain *A. butzierei* D2686 and SW-OL2 was obtained by insertion of the Aph-A3 kanamycin resistance cassette (1.5 kb) at the *BclI* site and introduced into strain *A. butzierei* D2686 and SW-OL2 genomes by homologous recombination using electroporation, selecting for kanamycin resistance. The *cadF* mutant strains were grown on *Arcobacter* agar with 15% (w/v) casamino acids (BIO 101) and 50 µg/ml kanamycin. Disruption of the *cadF* gene in each strain was also confirmed by PCR using K^{r} -specific primers described as KancasF and KancasR (Figure 3.21) after purifying the chromosomal DNA by using the gel purification technique in Section 2.2.10.

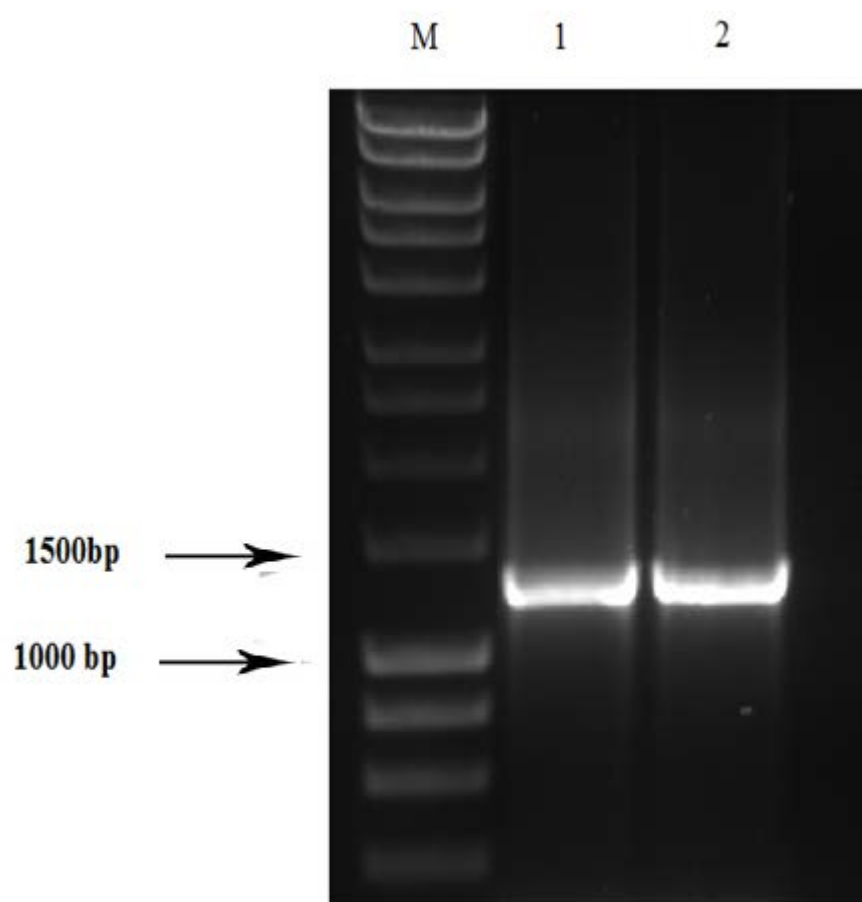


Figure 3.21 PCR profiles of the *aphA-3* gene in the *cadF* disruption mutants. Lane 1: *A. butzleri* D2686; Lane 2: SW-OL2; M: Hyperladder I DNA marker.

3.6. Adherence assay by differently *cadF*-expressing *Arcobacter* strains

To investigate the role of the CadF protein in the binding of *Arcobacter* strains to INT-407 cells, the interaction of wild-type *Arcobacter* and *cadF* mutant strains (both *A. butzleri* D2686 and SW-OL2) with INT-407 cells was examined by carrying out binding assays. In addition to testing the wild-type and *cadF* deficient strains, binding assays were also used to compare the adherence ability of clinical versus environmental *Arcobacter* isolates.

All *Arcobacter* strains were tested in two assays for their ability to adhere to INT-407 cells as described in Section 2.3.5.2. *E. coli* EMG 14 was included as positive control, and bacteria-free INT-407 cultures were used as negative controls. Adhesion results were expressed as the percentage of the number of bacteria (\pm SD) adhering to INT-407 cells (Table 3.6).

All the strains were able to adhere to INT-407 cells *in vitro*. The highest percentage adherence of wild-type *Arcobacter* (81%) was observed for *A. butzleri* strain D2686, which had originally been isolated from human faeces (with associated diarrhoea) and the lowest percentage (21%) was observed for *A. cryaerophilus* strain A169/B isolated from aborted bovine foetus (Table 3.6). The lowest number of bacteria adhered to wild-type *Arcobacter* was also observed for *A. cryaerophilus* strain A169/B (53 ± 2.52) and the highest number (64 ± 12.50) in *Arcobacter butzleri* strain D2686 (Table 3.6). There was a significant reduction ($\sim 50\%$) in adherence for *A. butzleri* D2686- $\Delta cadF$ ($P > 0.01$) and SW-OL2- $\Delta cadF$ ($P > 0.001$) mutant strains (Table 3.6, Figure 3.23).

These findings demonstrate that the CadF protein is an important cell adherence factor especially in *Arcobacter butzleri* and could be for all *Arcobacter* species. Furthermore, the strains that were tested for their adhesion capacity showed two distinct attachment patterns; diffuse and localized adherence. However, most *Arcobacter* strains in this study showed the diffuse pattern in which bacteria adhere evenly to the whole cell surface (Figure 3.22). Only the *Arcobacter skirrowii* 449/80 strain showed a localized adherence in which bacteria form characteristic micro-colonies on the surface of the INT-407 cell (Figure 3.22).

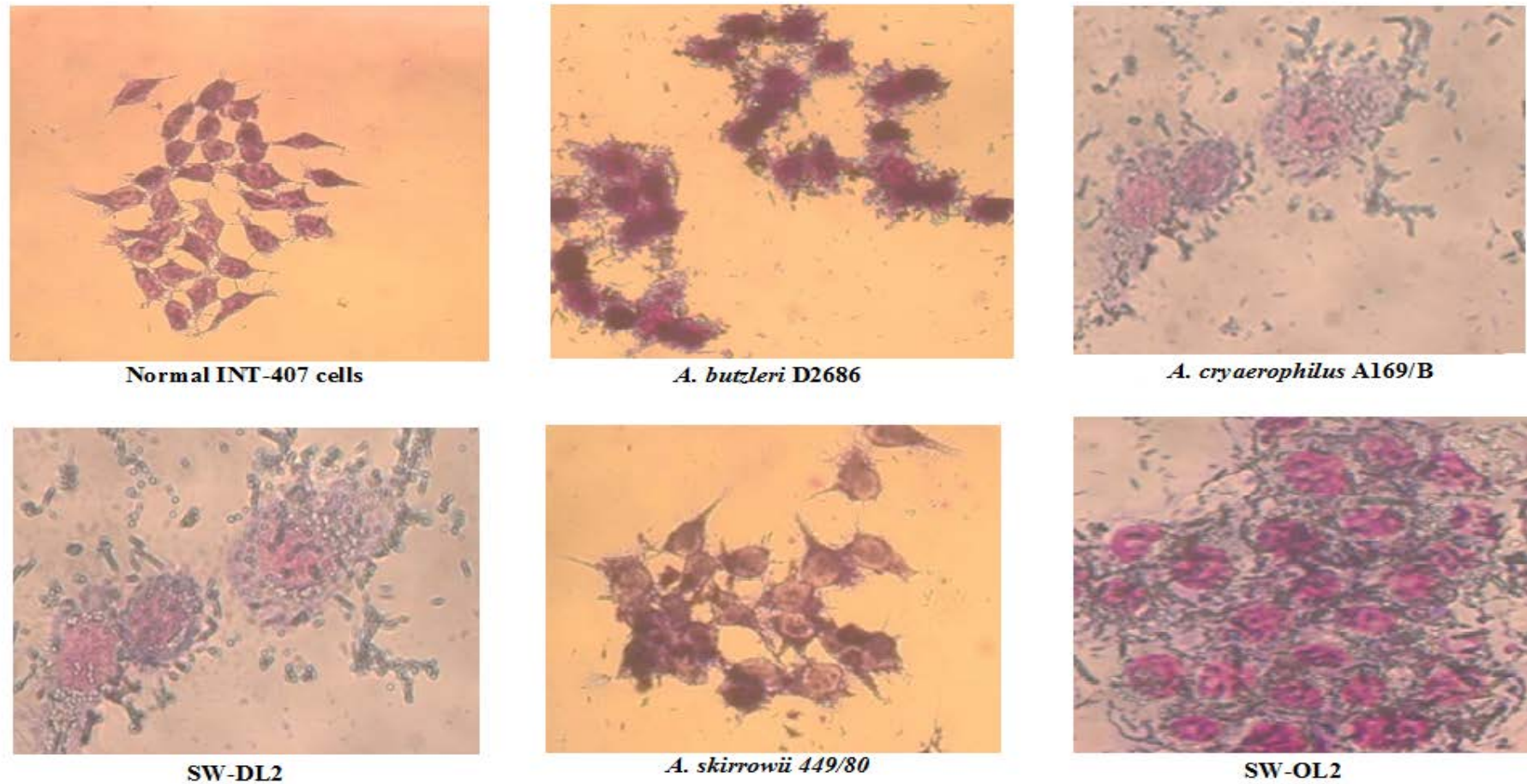


Figure 3.22 Microscopic images of uninfected and infected INT-407 cells with different *Arcobacter* strains

Table 3.6 Adhesive capacity of different strains of *Arcobacter*

Strain	Adhesion ¹	Number of adhered bacteria ²
<i>A. butzleri</i> D2686	81.39%	64 ±12.50
<i>A. cryaerophilus</i> A169/B	21.00%	53 ±2.52
<i>A. skirrowii</i> 449/80	42.30%	56 ±5.69
SW-DL2	72.60%	61 ±3.06
SW-OL2	76.42%	60 ±2.65
<i>A. butzleri</i> D2686- Δ <i>cadF</i>	16.28%	32 ±2.65
SW-OL2- Δ <i>cadF</i>	16.43%	29 ±3.51

¹ Range of the percentage of bacteria adhering to INT-407 cells.

² Adherence is expressed as the mean number of adherent bacteria to INT-407 cells ± standard deviation (n=3).

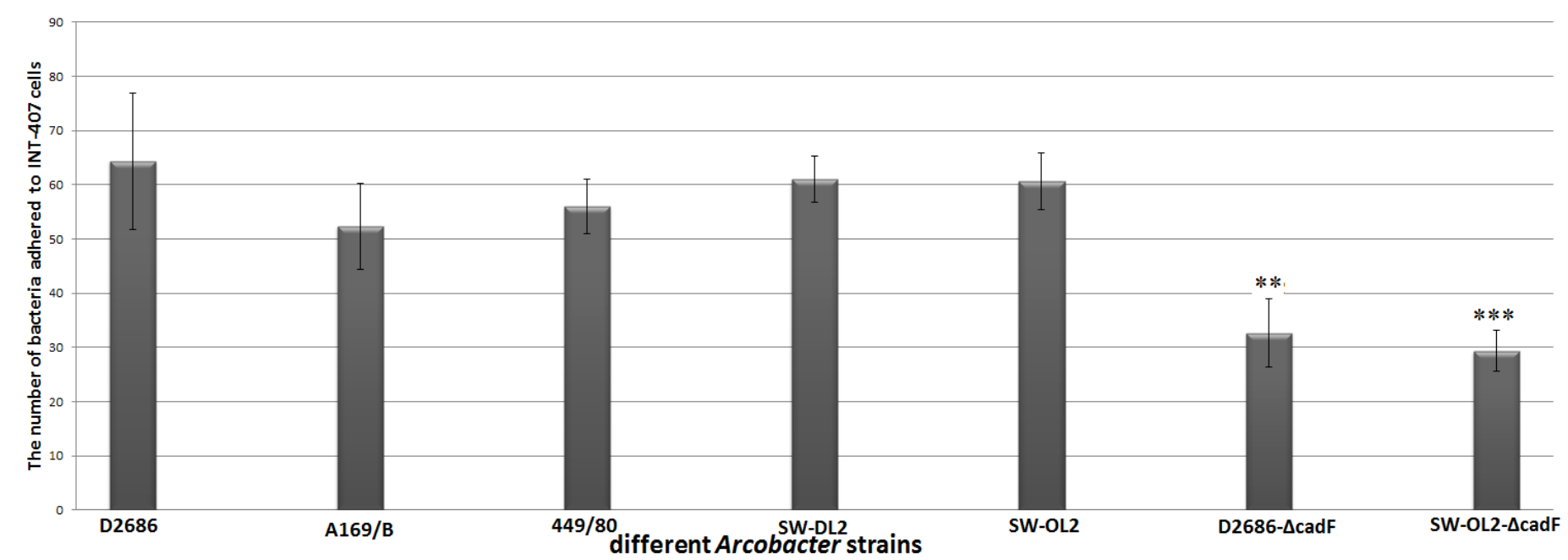


Figure 3.23 Adhesive capacity of different wild type strains of *Arcobacter* and *cadF* mutant strains

**Significant difference, $P < 0.01$

***Significant difference, $P < 0.001$

3.7. Analysis of *Arcobacter* DNA by slot blot hybridization

Slot blot hybridization was used to identify five putative virulence genes (*cadF*, *ciaB*, *flaA*, *flaB* and *pldA*) in all strains in this study. These genes have previously been identified in the *A. butzleri* strain RM4018, and might be involved in the ability of *Arcobacter* species to attach and invade the host cells particularly epithelial cells of the gastrointestinal tract. This involved synthesizing DIG-labelled probes to target *A. butzleri* genes, binding an anti-DIG antibody–alkaline phosphatase conjugate to the probe and using chemiluminescence to detect positive signals.

The specificity of the all probes used for the identification of the target genes in this study were validated using the BLAST programme available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. *A. butzleri* D2686 was used as the positive control and *Clostridium beijerinckii* (NCIMB 8052) as the negative control (Figure 3.24). Analysis of DNA-slot blots showed that the genes *cadF*, *ciaB*, *flaA*, *flaB* and *pldA* were present in all strains used in this study (Figure 3.24).

3.8. Analysis of expression of the *cadF* gene by slot blotting

The slot blotting technique was also used to estimate the reproducibility of *cadF* mRNA levels, and to provide an indication of expression levels of the *cadF* gene between clinical and environmental *Arcobacter* isolates.

Transcription of the *cadF* gene was analysed by hybridization of DIG-labelled PCR-amplified DNA probes against the mRNA of the *cadF* gene. Probes were hybridised with total RNA (~20 µg) extracted from bacterial cells grown on INT-407 cell lines. Semiquantitative measurements of *cadF* mRNA levels by slot blotting showed that mRNA related to the *cadF* gene was present in all strains; however, *A. cryaerophilus* A169/B and SW-OL2 gave a slightly stronger signal than other strains (Figure 3.25). *Pectinatus cerevisiiphilus* (DSM 20467) was used as negative control (provided as RNA sample from Dr W.J.Mitchell, HWU).

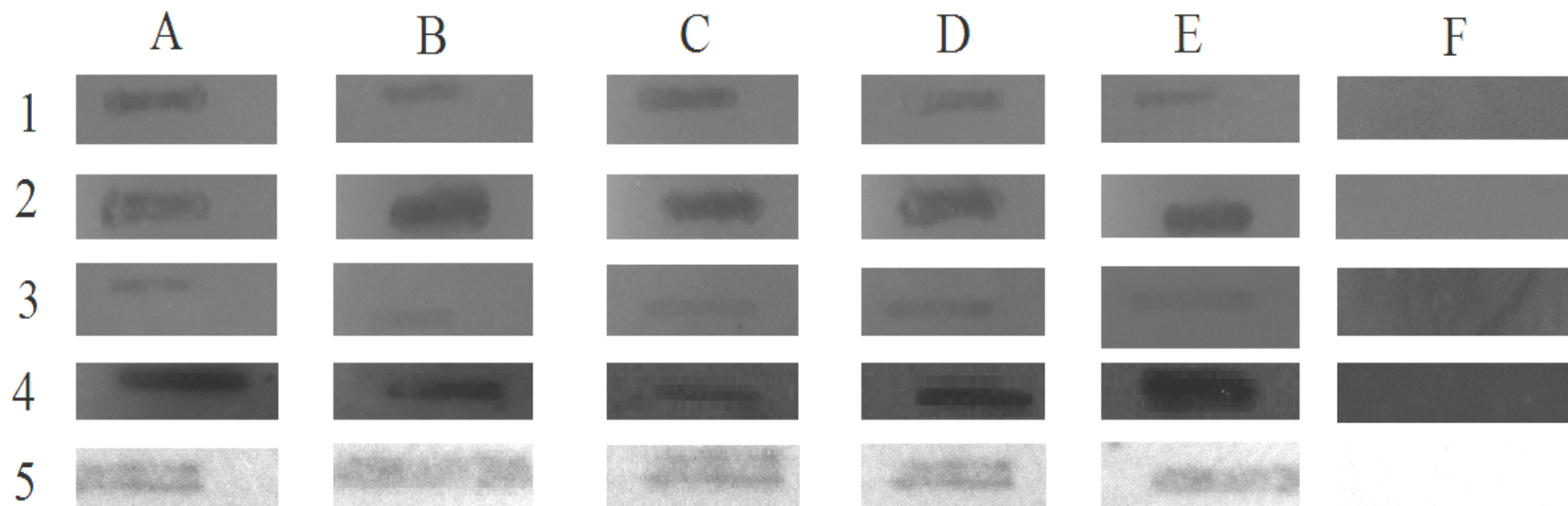


Figure 3.24 Slot blot hybridization of *Arcobacter* strains for the genes *cadF*, *ciaB*, *pldA*, *flaA* and *flaB*. (A): *A. butzleri* (D2686); (B): *A. cryaerophilus*; (C): *A. skirrowii*; (D): SW-DL2; (E): SW-OL2; (F): negative control, *Clostridium beijerinckii* (NCIMB 8052); (1): *cadF*; (2): *ciaB*; (3): *pldA*; (4): *flaA*; (5): *flaB*.



Figure 3.25 Detection of mRNA for *cadF* gene by slot blot hybridization. (1): *A. butzleri* D2686; (2): *A. cryaerophilus* A169/B; (3): *A. skirrowii* 449/80; (4): SW-DL2; (5): SW-OL2; (A): *Pectinatus cerevisiiphilus* (DSM 20467).

3.9. Relative gene expression of *cadF* with other putative virulence genes (*ciaB*, *flaA*, *flaB* and *pldA*) using Real-Time PCR

Based on the previous results of identification of five putative virulence genes in all *Arcobacter* strains in this study, the expression of *Arcobacter* putative virulence genes *cadF*, *ciaB*, *flaA*, *flaB* and *pldA*, was monitored during the infection of INT-407 cells. Comparison was also made between the expression of the *cadF* gene and other putative virulence genes in all clinical and environmental *Arcobacter* isolates in this study. The relative expression of each gene was determined three times in each of two experimental RNA samples (as treated and untreated samples) and was expressed as the fold difference. Positive control was reference gene (housekeeping gene; *glnA*) and negative control (ddH₂O) was also included. RNA quality and integrity is the most important factor affecting the outcome of these experiments and obtaining meaningful gene expression data; therefore, the purified total RNA was quantitated using a Shimadzu wide range UV spectrophotometer at multiple wavelengths between 240-320nm, and UVProbe software was also used to determine the purity of total RNA sample. Electrophoresis of RNA samples can be used to check for genomic DNA contamination and also for RNA degradation (Figure 3.26).

3.9.1. RT-PCR efficiency

For each gene, a cDNA dilution curve was generated and real-time PCR was performed to calculate the efficiency (E) of the PCR. The corresponding real-time PCR efficiency was calculated according to the equation: PCR efficiency (E) = $-1 + 10^{(-1/\text{slope})}$ and amplification efficiency was calculated using the equation $= 10^{(-1/\text{slope})}$. The PCR efficiencies of all genes should be preferably at or above 90% and a slope of -3.32 indicates the PCR reaction is 100% efficient. Slopes in the range of -3.60 to -3.10 are generally considered acceptable for real-time PCR and these slope values correlate to amplification efficiencies between 90% (1.9) and 110% (2.1). The absolute method was preformed to measure this by performing a 10-fold serial dilution for each cDNA of each gene and plotting the C_T as a function of log (10) concentration of template.

The PCR efficiencies were 102%, 99%, 105%, 114%, 90% and 109% (Figure 3.27), while the amplification efficiencies were 2.02, 1.99, 2.05, 2.14, 1.90 and 2.09 (for *cadF*,

ciaB, *pldA*, *flaA*, *flaB* and *glnA*, respectively). The linear correlation coefficient (R^2) was also performed for all genes and ranged from 0.99-1.00.

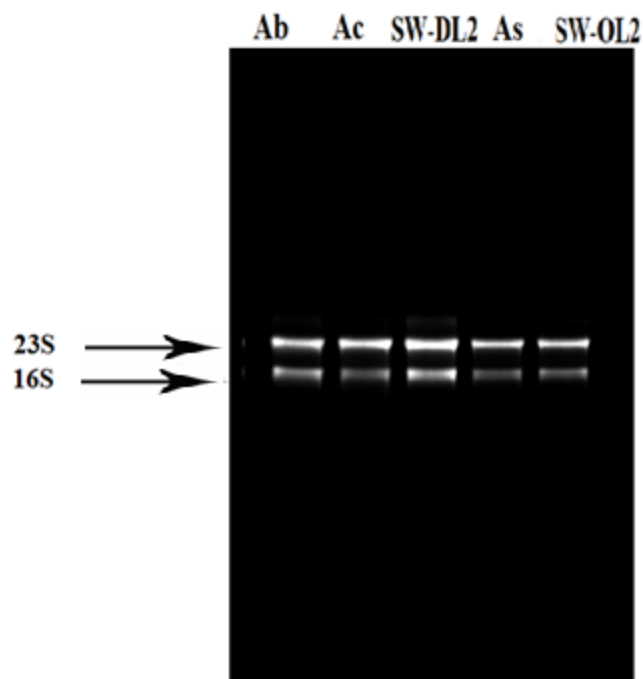


Figure 3.26 Total RNA extract from tissue culture using lysis technique with RNeasy Mini kit (QIAGEN). Ab: *A. butzleri* D2686; Ac: *A. cryaerophilus* A169/B; As: *A. skirrowii* 449/80.

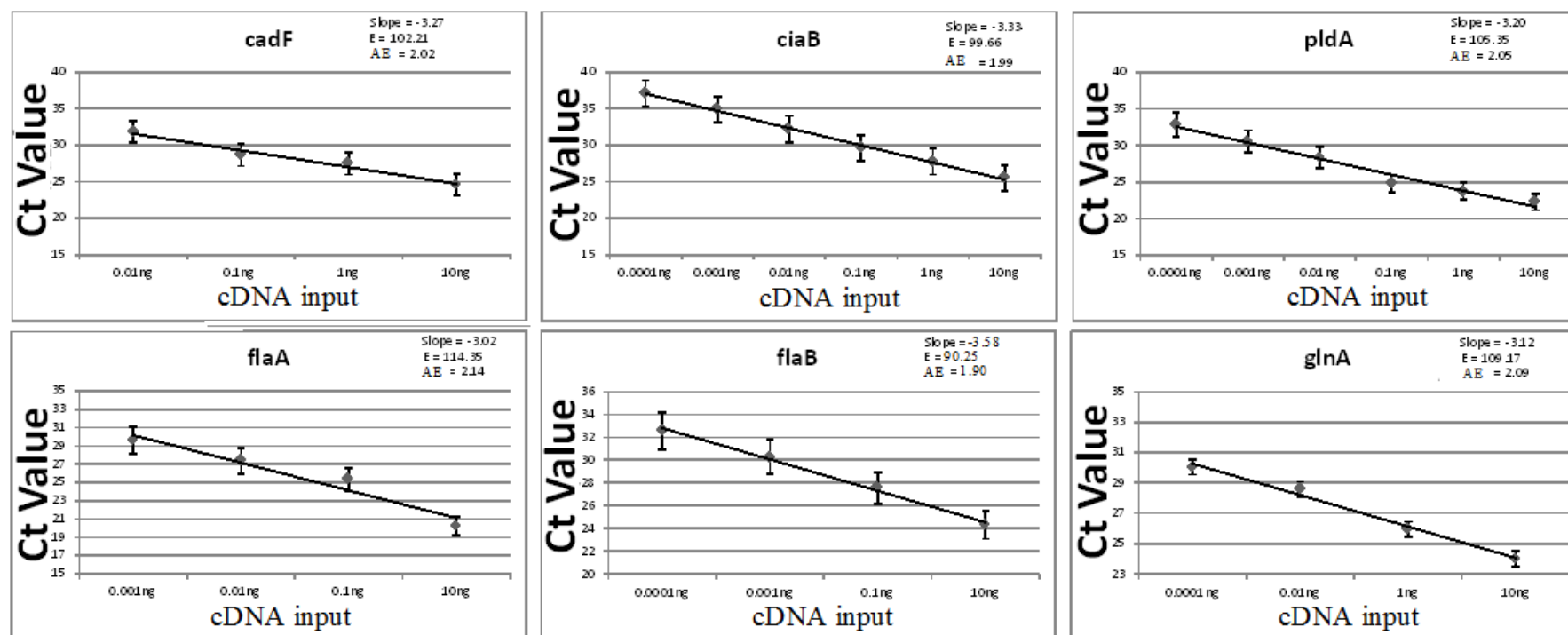


Figure 3.27 Determination of qPCR efficiencies of target genes (*cadF*, *ciaB*, *pldA*, *flaA* and *flaB*) and reference gene (*glnA*). The formula for this calculation is $\text{Efficiency} = -1 + 10^{(-1/\text{slope})}$ and Amplification efficiency (AE) was also been calculated using the equation $\text{AE} = 10^{(-1/\text{slope})}$.

3.9.2. The analysis of relative gene expression data of RT-PCR analysis

RT-PCR analysis of gene expression of *cadF* relative to the other putative virulence genes (*ciaB*, *pldA*, *flaA* and *flaB*) was determined after 3h incubation of each *Arcobacter* strain with INT-407 cell line at 37°C. Quantitative values were obtained by using the comparative threshold cycle ($\Delta\Delta C_T$) method recommended by Applied Biosystems. The C_T value corresponds to the PCR cycle at which the first detectable increase in fluorescence associated with the exponential growth of PCR products occurs. The relative expression of each gene was determined three times in each of the two experimental RNA samples and was expressed as the fold difference.

Generally, the expression levels of the five genes studied were significantly upregulated in all strains examined. RT-PCR analysis revealed that the *cadF* gene was expressed at a higher level in *A. cryaerophilus* strain A169/B and *A. skirrowii* strain 449/80 (Figure 3.28) when compared to the three other strains (D2686, SW-DL2 and SW-OL2). Therefore, the analysis of relative gene expression data of the strains showed that CadF protein may be an important virulence factor contributing to adhesion to epithelial cells and subsequent induction of disease. The four other putative virulence genes (*ciaB*, *flaA*, *flaB* and *pldA*) showed significant differences in their transcript levels (Figure 3.29). The *pldA* gene was poorly expressed in SW-DL2 although it was expressed at a higher level in other strains (Table 3.7). Furthermore, flagellar gene (*flaA*) was expressed at a higher level in *A. butzleri* strain D2686 and SW-DL2 when compared to the other three strains (*A. cryaerophilus* strain A169/B, *A. skirrowii* 449/80 and SW-OL2).

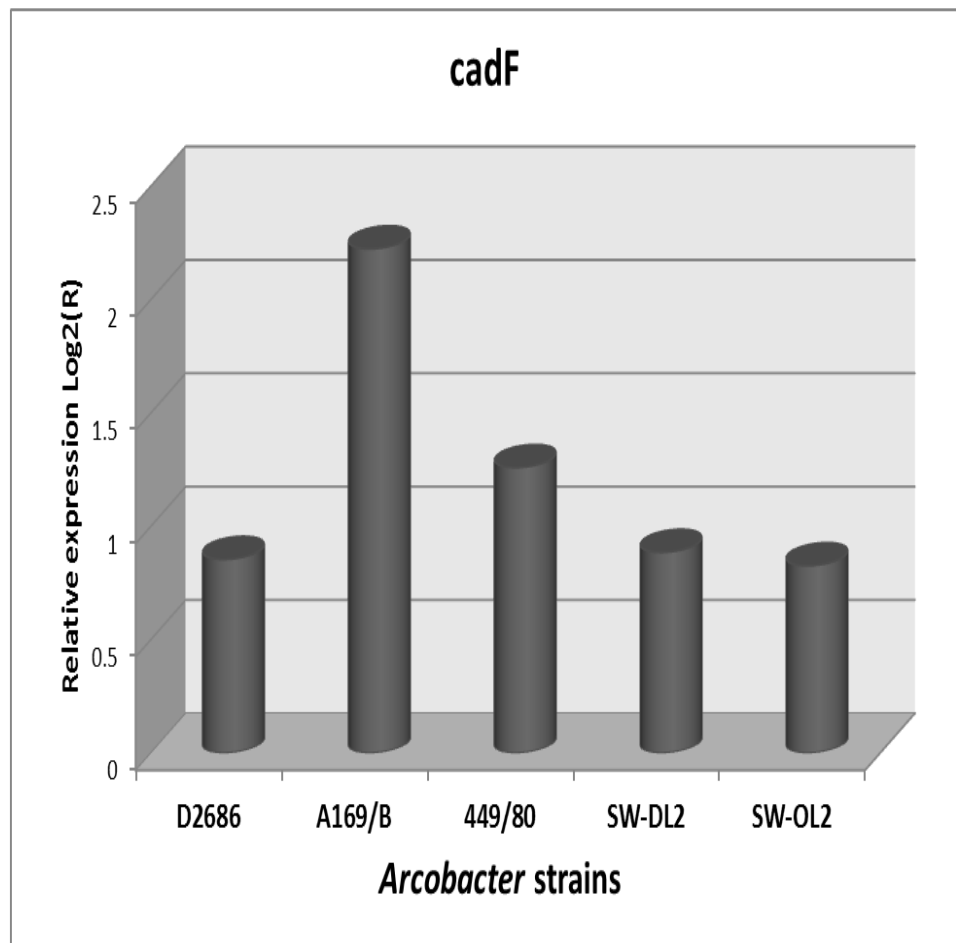
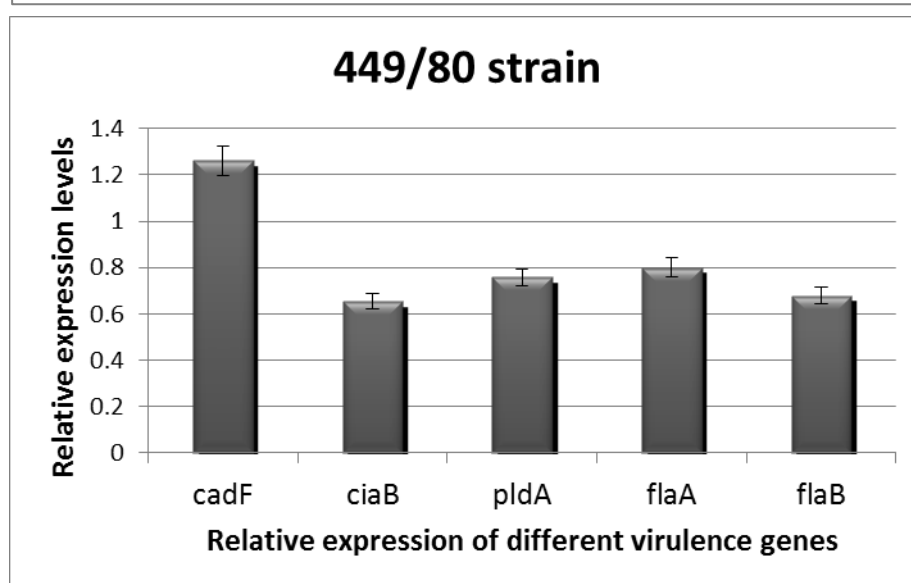
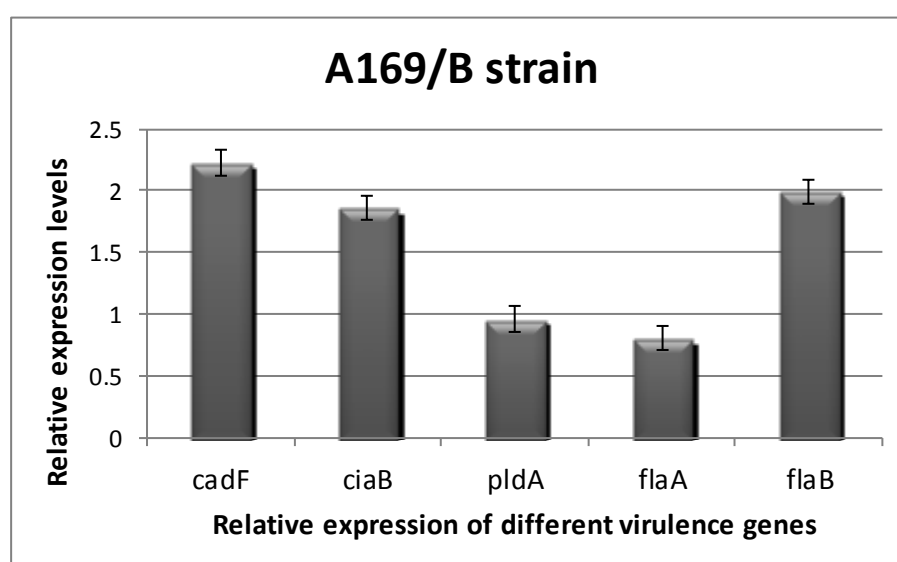
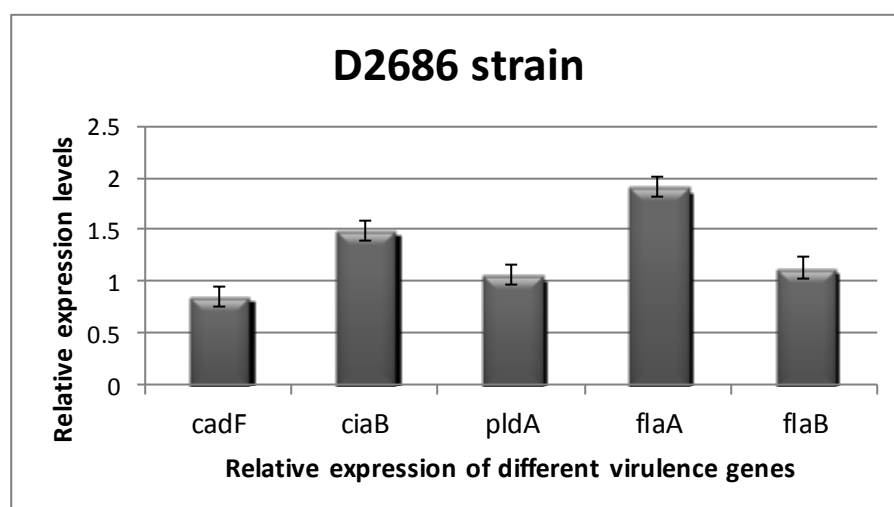


Figure 3.28 The comparison of the *cadF* transcription for all studied *Arcobacter* strains in this study. D2686: *A. butzleri*; A169/80: *A. cryaerophilus*; 449/80: *A. skirrowii*.



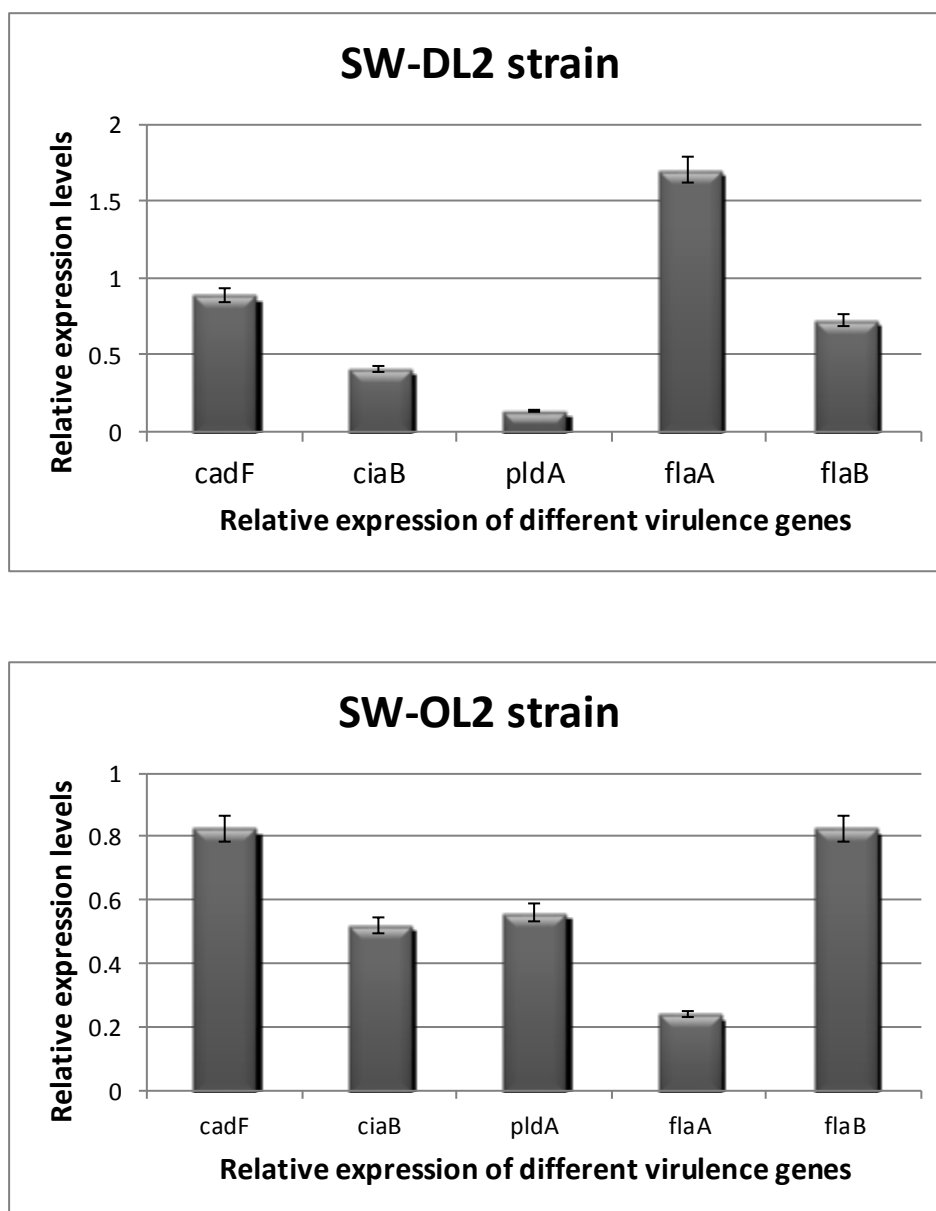


Figure 3.29 The relative expression levels for five putative virulence genes during invasion of INT-407 cell with studied *Arcobacter* strains. D2686: *A. butzleri*; A169/B: *A. cryaerophilus*; 449/80: *A. skirrowii*.

Table 3.7 Relative expression levels of different virulence genes in response to trans-infection of INT-407 cell lines.

Gene	Gene production or function	Relative expression and Induction ratio	
		Strain	Relative expression [Log ₂ (R)]
<i>cadF</i>	Fibronectin-binding protein (FnBP), adherence	D2686	0.855
		SW-OL2	0.827
		SW-DL2	0.886
		A169/B	2.225
		449/80	1.260
<i>ciaB</i>	Campylobacter invasion antigen A, invasion and adherence	D2686	1.495
		SW-OL2	0.407
		SW-DL2	0.520
		A169/B	1.860
		449/80	0.654
<i>flaA</i>	Flagellin A, chemotaxis and adherence	D2686	1.199
		SW-OL2	1.701
		SW-DL2	0.240
		A169/B	0.801
		449/80	0.801
<i>flaB</i>	Flagellin B, chemotaxis and adherence	D2686	1.132
		SW-OL2	0.723
		SW-DL2	0.827
		A169/B	1.994
		449/80	0.679
<i>pladA</i>	Phospholipase A, invasion and adherence	D2686	1.061
		SW-OL2	0.133
		SW-DL2	0.559
		A169/B	0.959
		449/80	0.757

CHAPTER 4 DISCUSSION

4. Discussion

In the last few decades, several emerging water and food-borne pathogens have been shown to have a significant impact on public health. This has led to scientific and political efforts to address these issues, which have also had a negative effect on the economy. This project addressed one of the emerging water and food-borne bacterial pathogens, *Arcobacter* spp., which were classified previously as ‘aerotolerant campylobacters’. Increasing numbers of reports on arcobacters during the last few years have highlighted the significance of these organisms as a potential food safety problem. This is combined with a growing awareness of arcobacters as emerging water and foodborne pathogens (Atabay *et al.*, 2006). In recent decades, *Arcobacter* species have become significantly more important in both veterinary and human public health because they are potential zoonotic bacteria (Collado *et al.*, 2011; Houf *et al.*, 2005). Disease caused by organisms of the genus *Arcobacter* is still likely to be underestimated and the mechanisms by which the bacteria cause disease in animals and humans are still largely unknown because of the insufficiency in identification methods (Vandenberg *et al.*, 2004).

Physiologically, *Arcobacter* species differ significantly from *Campylobacter* and *Helicobacter* in being both aerotolerant and capable of growth at 15 °C, attributes which could give them a considerable advantage when it comes to water and foodborne transmission. However, the evidence for an association between *Arcobacter* and diarrhoeal diseases in humans and animals remains circumstantial at present. Since the complete genome of *Arcobacter butzleri* strain RM4018, isolated as a human clinical strain, has been published by Miller *et al* (2007), special emphasis has been placed on data about the physiology and genetics of this genus. *Arcobacter butzleri* is the most important species of the genus *Arcobacter* and has been categorized as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002).

The rates of isolation of *Arcobacter* species from the environment vary widely from 0.5 to 97% (Martin and Adams, 2010) and many studies have shown the presence of *Arcobacter* species in various type of water such as surface water, ground water and drinking water (Moreno *et al.*, 2003; Morita *et al.*, 2004; Diergaardt *et al.*, 2004; Rice *et*

al., 1999; Jacob *et al.*, 1993). It has been suggested that water can play an important role in the transmission of *Arcobacter* species and drinking water has been cited as a major risk factor for many diseases in humans. *Arcobacter* species have been widely detected in freshwater in many countries such as Japan, Thailand, South Africa, Germany and USA (Cheonghoon *et al.*, 2012; Morita *et al.*, 2004; Diergaard *et al.*, 2004; Jacob *et al.*, 1998).

The first part of this study (Section 3.1) demonstrated that only two out of eleven Scottish surface water sources were contaminated with *Arcobacter* (18%) and two water-associated *Arcobacter* isolates were obtained from these sources (Duddingston Loch and Loch Ore). These were both shown to be closely related to *Arcobacter butzleri* (Section 3.1.4).

Although this is the first formally documented isolation of *Arcobacter* species from surface waters in Scotland (specifically Lothian and Fife regions), finding arcobacters in surface waters is consistent with the results published by other research groups. Indeed, *Arcobacter* species have been isolated from various types of surface water and also from contaminated ground water in several different countries (Stampi *et al.*, 1993; Rice *et al.*, 1999). Few studies on *Arcobacter* spp. and their isolation have been carried out in the UK in comparison with other countries. This study has focussed on the Lothian and Fife regions, which together form only a small percentage of the water sources in Scotland and thus, further studies are needed to accurately detect the extent of *Arcobacter* contamination in Scottish waters.

It is likely that the presence of many different wild birds around these two lochs might have led to the contamination of these water sources by arcobacters, although there is no direct evidence so far that birds could be a reservoir for arcobacters in Scotland. Animal faecal contamination has also been proposed as a means of introducing *Arcobacter* species into different water sources, leading to colonisation of *Arcobacter* in both animals and humans (Donachie *et al.*, 2005; Snelling *et al.*, 2006). It has also been hypothesized that the sources of surface water contamination by these bacteria could be from the faeces of livestock animals and from farm effluents (Chinivasagam *et al.*, 2007). Indeed, several studies have shown that *Arcobacter* species can be found in

surface water mostly contaminated by faecal materials (Cheonghoon *et al.*, 2012; Fong *et al.*, 2007).

Arcobacter's capacity to survive in water is influenced by multiple factors such as the presence of organic matter in the water, but also by water temperature, turbidity and pH (Van Driessche and Houf, 2008; Cheonghoon *et al.*, 2012). Water temperature negatively correlates to *Arcobacter* contamination as shown in previous research studies that demonstrate that *Arcobacter* can grow at temperatures of 15 °C or even lower (Fera *et al.*, 2010; Cheonghoon *et al.*, 2012). *Arcobacter* has a demonstrated susceptibility to chlorine (Rice *et al.*, 1999; Moreno *et al.*, 2004), but it is still unknown whether or not conventional procedures for treating drinking water can effectively remove these bacteria, as discussed by Ho *et al.* (2006a). In a recent study conducted by Collado and Figueras (2011), it was found that although *A. butzleri* and *A. cryaerophilus* were prevalent in river water, they were never isolated from the finished purified product, evidently suggesting that purification and treatment of water are both effective in removing *Arcobacter* from water sources. Nonetheless, as has been discussed earlier, the ability to eliminate *Arcobacter* during the treatment and processing that produces drinking water, has been poorly studied. Further investigations are necessary to successfully conclude whether the conventional procedures used need to be assessed to determine their ability to eliminate these bacteria or whether they can continue to be used without additional development.

The ability of bacteria to adhere to surfaces and also form biofilms has been associated with bacterial virulence and environmental survival (Gaynor *et al.*, 2007). It has been demonstrated that organisms belonging to the genus *Arcobacter* have the capacity to adhere to pipes and form biofilms on various pipe surfaces, such as stainless steel, copper, and plastic and therefore can readily colonize water distribution systems (Welsh *et al.*, 2011; Fernandez *et al.*, 2008; Assanta *et al.*, 2002). The capacity of *Arcobacter* to adhere to pipes and form biofilms has also been considered a major factor in water contamination (Shah *et al.*, 2011) and the continued proliferation of the organisms in the slaughterhouse environment is largely due to its ability to form a biofilm under chilled conditions (Kjeldgaard *et al.*, 2009). Recently, Susana *et al.* (2013) have revealed that 26 strains presented biofilm-forming ability, which might in part explain the prevalence

and survival of *Arcobacter* in the slaughterhouse environment. However, studies on biofilm formation by *Arcobacter* species are still limited, and considerable work is required to evaluate its true importance as a virulence determinant and its effect in the survival and spread of these bacteria (Kjeldgaard *et al.*, 2009).

In the current study, *Arcobacter butzleri* was the only species found in the surface waters (from Fife and Lothian regions; Section 3.1), which might be due to the limited number of samples taken in this study. This observation does, however, agree with other studies that have found *A. butzleri* to be the most prevalent of the *Arcobacter* species, followed by *A. cryaerophilus*. The genetic diversity of these bacteria in samples of surface waters is yet to be explored and it is still unconfirmed whether or not specific genotypes are able to persist in water. It has been noted that ambient temperature may contribute to seasonal variation of *Arcobacter*, although no winter or autumn samples were taken in the current study (April-August) and so seasonal variations of *Arcobacter* populations cannot be determined in this study. Previous studies have found that recovery rates at all sites were greater in the spring and summer (Collado *et al.*, 2010; Andersen *et al.*, 2007; Stampi *et al.*, 1999) but further investigation is required.

Although numerous methods are available to isolate *Arcobacter* species, difficulties have been faced in their isolation for a variety of reasons that have been reported previously. The main problem is that *Arcobacter* species have a low rate of growth on synthetic media and are easily outgrown by other contaminating microorganisms in the environmental and clinical samples tested. In this study, *Arcobacter* was found to take extended times to grow during the isolation and characterisation experiments, but more especially when using the type strains to show their pathogenicity. The recovery and isolation of arcobacters was made more difficult in this study because of the number of other species present in the water samples. Selective media were used in combination with microaerobic conditions with some success although non-target organisms grew even under microaerophilic conditions (Section 3.1.1). The use of recommended antibiotics like CAT and CCDA reduced but did not prevent the growth of contaminating microorganisms, especially in the specimens isolated from water. In this study it was noted that arcobacters grow slightly faster on blood agar (Section 3.1.1), producing small white colonies that are a little larger and easier to see than the

transparent colonies that grow on other types of media, and this may be effectively included in a strategy to optimise isolation of the organisms.

The phenotypic identification of *Arcobacter* species is not straightforward and uncharacteristic reactions are not uncommon. Aberrant results with *Arcobacter* isolates are attributed to the low metabolic activity of the species (Atabay *et al.*, 2006; On, 1996), and as a consequence a reliable identification scheme does not yet exist (Maria *et al.*, 2008). The characteristics of arcobacters were variable for some of the biochemical tests that were used, including indoxyl acetate hydrolysis (Table 3.2), a result that agrees with recent work published by Collado *et al.*, 2009a, whose study highlighted that not all *Arcobacter* species give positive results with this test. Consequently, the biochemical tests are not a robust method for unequivocal identification of *Arcobacter* species.

Amplification of 16S and 23S rDNA genes seemed to be the ideal method to detect arcobacters (Houf *et al.*, 2000), but this method also has some limitations since the primers used allow characterisation of only three species of *Arcobacter* (*A. butzleri*, *A. cryaerophilus* and *A. skirrowii*). 27 water isolates were tested using PCR amplification using the set of species-specific primers described by Houf *et al.* (2000). Using genomic DNA template, 18 isolates generated end-products using the *Arcobacter butzleri* primers (Section 3.1.2). None of the isolates produced amplification products with either the *A. skirrowii* or *A. cryaerophilus* primers, although successful amplification was seen using positive DNA controls from reference strains of these organisms. This technique allowed the preliminary identification of the water isolates as *A. butzleri* and in order to confirm this, the 16S rRNA genes of the 18 isolates were sequenced. Sequencing and BLAST analysis showed only two of these isolates had significant homology (92-96% identity) to *A. butzleri* (Section 3.1.4). RAPD analysis was performed to distinguish between these isolates. The primers initially developed by Houf *et al.* (2000), which were used in this study, are now accepted as having substantial limitations in their ability to identify the wide range of *Arcobacter* spp. currently recognised. Doudah *et al.* (2010) have developed Houf's methodology and introduced a multiple-PCR assay with seven primers for the more accurate identification of more clinical and environmental species of *Arcobacter* (Doudah *et al.*, 2010). For

the purposes of this study, the two *A. butzleri* water isolates identified (SW-DL2 and SW-OL2) were selected for further study, but the remaining 16 isolates may also contain other arcobacters of interest, if the extended range of primers introduced by Doudiah *et al.* (2010) were used in their analysis.

Several other DNA-based approaches have been described for characterizing species of *Arcobacter*. These include, 16S rDNA-RFLP (Maria *et al.*, 2010), RFLP (Marshall *et al.*, 1999; Hurtado and Owen, 1997; Carderelli *et al.*, 1996; Kiehlbauch *et al.*, 1991), sequencing of 16S rRNA (Lau *et al.*, 2002), sequencing of genes including *rpoB-rpoC* and *gyrA* (Morita *et al.*, 2004; Abdelbaqi *et al.*, 2007b), multiplex-PCR techniques (Houf *et al.*, 2000; Doudiah *et al.*, 2010), real-time PCR (Brightwell *et al.*, 2007), a microarray technique (Quinones *et al.*, 2007) and real-time fluorescence resonance energy transfer PCR (Abdelbaqi *et al.*, 2007b). While all of these techniques have been applied to the detection and identification of *Arcobacter* species, none are able to discriminate all of the accepted species of *Arcobacter* (Maria *et al.*, 2008; Doudiah *et al.*, 2010).

The studies described in this thesis clearly demonstrate that the sequencing of 16S rDNA was the best technique employed to identify *Arcobacter* at the genus level. Nonetheless, traditional phenotypic tests have their use in narrowing down the number of bacterial isolates from environmental samples. Although the cost effectiveness of using 16S rRNA gene sequencing in routine clinical microbiology laboratories remains to be evaluated, in this project 16S rDNA gene sequencing proved to be useful for the identification of *Arcobacter* species from environmental samples which exhibited ambiguous biochemical tests. The use of 16S rDNA gene sequencing will continue to be the gold standard for the identification of bacteria, particularly in relation to *Arcobacter* species, for the foreseeable future, or until replaced by new methodologies that may be put into routine use in clinical microbiology laboratories and that would replace traditional phenotypic testing.

In this project, most of the *Arcobacter* clinical (reference) strains and the environmental isolates showed positive elastase activity (Section 3.2). This is significant because elastase is considered to be a virulence factor, which causes disruption of the tight

junctions of connective tissue (Nicas *et al.*, 1985) and its role as a virulence determinant in other pathogens including *Staphylococcus aureus*, *Erwinia carotovora*, and *Pseudomonas aeruginosa* has been reported. It is also considered to have a role in bacterial colonisation (Kim *et al.*, 2000) and, more generally, elastases have been shown to play a pathologic role in pulmonary emphysema, cystic fibrosis, infections, inflammation and atherosclerosis (Bieth, 2001). Elastase has a tissue-damaging activity and is capable of degrading various plasma proteins such as immunoglobulins, coagulation and proteinase inhibitor (Bengt and Olgerts, 1983). The three reference strains tested (*A. butzleri* D2686, *A. cryaerophilus* A169/B, and *A. skirrowii* 449/80) showed the production of elastase enzyme. Several studies demonstrated that elastase can proteolyze a variety of host proteins such as immunoglobulins and complement factor (Hassett *et al.*, 1992; Hong and Berhane, 1992).

Several research studies have shown that *Arcobacter* species (including *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*) show variable alpha-haemolytic activity on blood agar plates (Atabay *et al.*, 1998; 2003; 2006; 2008; On *et al.*, 1996; Snelling *et al.*, 2006; Hye *et al.*, 2010). In this study the reference *Arcobacter* strains and water isolates were similarly tested for growth and haemolysis on blood agar supplemented by four types of erythrocytes (human, cattle, sheep and chicken). No haemolysis was observed for any of the target strains tested (Section 3.2).

Based on the genomic sequence analysis of *A. butzleri* RM4018, there is good evidence that haemolytic genes may be encoded by this species. Consequently further investigation was undertaken to confirm whether, if concentrated ECP was isolated, that it might show activity not seen from the slow-growing bacterial cultures. ECP was isolated from each of the reference and water strains and used in the haemolytic assay (Section 3.3). The results showed that all ECP preparations of *Arcobacter* strains caused haemolysis of chicken and sheep erythrocytes. One strain (*A. butzleri* D2686) was also able to haemolyse human erythrocytes, but no haemolysis of erythrocytes from cattle or horse was observed by the ECPs from any strain.

From the study of Tsang *et al.* (1996), two strains of *Arcobacter* were shown to agglutinate erythrocytes from human (blood group A and O), sheep and rabbit. Both

Arcobacter strains gave strong macroscopic haemagglutination with erythrocyte suspension however, this ability was destroyed by heat and proteolytic enzyme treatment (Raymond *et al.*, 1996). The whole genome sequence of *A. butzleri* RM 4018, suggests the presence of putative genes that could encode haemolysins such as *tlyA* which encodes bifunctional riboflavin kinase/FAD synthase RibF. Another novel virulence determinant, HecA, is a member of the filamentous haemagglutinin (FHA) family, and *hecB* encodes a related haemolysin activation protein (Miller *et al.*, 2007). Furthermore, *Arcobacter* sp. L. also has two genes encoding putative haemolysin-activated secretion proteins (ABLL-0715, 0716) and *Arcobacter butzleri* ED-1 contains four genes (ABED-0889, 0985, 0491, 1668) that could produce putative-haemolysin activated proteins and haemolysin (Toh *et al.*, 2011).

In the present study, whole cell protein, outer membrane protein and ECP profiles generated by SDS-PAGE of three clinical *Arcobacter* (reference strains) and environmental isolates (SW-DL2 and SW-OL2) were compared in an attempt to distinguish virulent and environmental strains of *Arcobacter* species (Section 3.5). Overall, the whole cell protein profiles were very similar for most strains with the region between 30-230 kDa having more than 20 protein bands resolved including a major band at ~40 kDa. In contrast, the outer membrane protein profiles were more varied than was seen for the whole cell protein profiles. In the case of the outer membrane proteins, more than 10 bands could be seen for each strain, with major bands for *A. butzleri* D2686 of 34.6 kDa, *A. cryaerophilus* A169/B of 42.7 kDa, *A. skirrowii* 449/80 of 42.7 kDa, SW-DL2 of 97.2 kDa and SW-OL2 of 98.2 kDa. The outer membrane protein profiles were therefore too diverse to allow comparison between the strains. In general, the ECP profiles showed a very high level of similarity between *A. butzleri* and the two water isolates (SW-DL2 and SW-OL2), and between *A. cryaerophilus* A169/B and *A. skirrowii* 449/80. Overall, in this case, the protein profiles could not be used to easily discriminate the pathogenic reference strain *A. butzleri* D2686 from the water isolates, which is in agreement with other previous studies (Helena *et al.*, 2005; Janos, 2006).

Intestinal colonisation is a complicated process in which pathogens in the intestinal lumen penetrate the mucous barrier and adhere to the mucosal surface of the gut

enterocytes, increasing in cell number and later detaching to disseminate in the body (Benitez *et al.*, 1997). Bacteria that have the ability to attach to the host tissues can persist on the surface of the host cells or can be internalized into an intracellular compartment, allowing the bacteria to escape host defence mechanisms and remain in the host and attack tissues surrounding the initial site of colonization (Per Klemm *et al.*, 2000). Consequently, bacterial adherence to host tissues represents a potential target for the improvement of new antimicrobial agents (Danny *et al.*, 1999). It has been suggested that an understanding of the mechanisms by which bacteria adhere to host cells as well as characterising the adhesion factors and the host receptors are all important approaches to the prevention of serious bacterial infections. Purification of adhesins or receptor materials, management of sublethal dosage of antibiotics that suppress the formation and expression of bacterial adhesins and development of vaccines against bacterial adhesins are approaches that are being actively researched (Morgan *et al.*, 1978; Beachey, 1981).

Generally, most *Arcobacter* strains are able to colonize the intestinal mucosa and cause gastrointestinal disorders (Ho *et al.*, 2007). A few studies on the adhesive ability of pathogenic *Arcobacter* strains have been carried out in different mammalian cell lines (Fernandez *et al.*, 1995; Musmanno *et al.*, 1997; Carbone *et al.*, 2003; Vandenberg *et al.*, 2004; Ho *et al.*, 2007). To extend these observations, this project investigated the mechanisms by which *Arcobacter* strains adhere to intestinal epithelial cells (Section 3.6). The adherence assays in this study were performed as described in section 3.5.2. They investigated the ability of *Arcobacter* strains to attach to the INT-407 cells and also determined the role of the CadF protein in the binding of *Arcobacter* through the interaction of both the wild-type *Arcobacter* and *cadF* mutant strains (in both *A. butzleri* D2686 and SW-OL2).

All reference *Arcobacter* strains and environmental isolates in this study were clearly able to adhere to INT-407 cells, with highest levels of adherence to INT-407 cells by *Arcobacter butzleri* strain D2686 (originally isolated from human faeces with diarrhoea), followed by the two water isolates (closely related to *A. butzleri*), and lower levels by *A. skirrowii* and *A. cryaerophilus*. These results are in agreement with other studies including that of Carbone *et al.* (2003), which tested 17 individual

environmental *Arcobacter butzleri* isolates for their adhesive capacity to Hep-2 and Hela line cells. The adhesion indices ranged from a weak value of 2.4 to a strong index of 28.0 and only six of the isolates were able to adhere strongly to both cell lines (Carbone *et al.*, 2003). In other studies, 12 *A. butzleri* strains isolated from human stool specimens were shown to be capable of adhering to Hep-2 cells (Vandenberg *et al.*, 2004). Four *Arcobacter* species (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius*) were tested for their ability to adhere to human Caco-2 and porcine IPI-2I cells, and all eight strains consistently showed the ability to adhere to both cell lines (Ho *et al.*, 2007).

The pathogenicity of bacteria commonly causing gastroenteritis is dependent on their ability to attach to and invade the human intestine (Konekel *et al.*, 1999; Svanborg-Eden *et al.*, 1976; Smith & Linggood, 1972). One of the main adhesins that *Campylobacter jejuni* uses to attach to mammalian cells is CadF which binds to fibronectin (Konkel *et al.*, 1997). The importance of CadF for the binding of *C. jejuni* to epithelial cells has been assessed *in vivo* (Ziprin *et al.*, 1999) and *in vitro* (Konkel *et al.*, 1997). The *cadF* genes from *C. jejuni* and *C. coli* specimens encode a 37-kDa outer membrane protein that promotes the organisms' binding to fibronectin (Konkel *et al.*, 1999a; Konkel *et al.*, 1997).

In this study, the *cadF* gene was selected because the sequence of this gene from the *Arcobacter butzleri* type strain RM4018 is closer to that of *Campylobacter jejuni* and *Wolinella succinogenes* than other species (Miller *et al.*, 2007). *Arcobacter butzleri* RM4018 also has sequences with homology to the *C. jejuni* genes that encode fibronectin-binding proteins, other outer membrane proteins and related peptidoglycan-associated (lipo) proteins.

To determine the overall contribution of the putative CadF protein in the binding of *Arcobacter* strains to INT-407 cells and the interactions of *cadF* mutant strains, cells were examined using a binding assay (Section 3.6). The present study has found a significant reduction (~50%) in adherence of *A. butzleri* D2686- Δ *cadF* ($P > 0.01$) and SW-OL2- Δ *cadF* ($P > 0.001$) mutant strains (Table 3.5; Figure 3.23). These findings demonstrate that the CadF protein is an important cell adherence factor at least in the

species of *Arcobacter butzleri* tested, and might be for other *Arcobacter* species. These data agree with previous studies for *Campylobacter jejuni* that show significant reductions (in some cases of up to 50-90%) in adherence to epithelial cells *in vivo* and *in vitro* of *C. jejuni cadF* mutants (Malgorzata *et al.*, 2007; Konkel *et al.*, 1997, 1999a, 2005; Marshall *et al.*, 2003).

The biological significance of the CadF adhesin has been demonstrated *in vivo* and it has been reported that a *C. jejuni* F38011 *cadF* mutant is unable to colonize the intestinal tract of Leghorn chickens (Ziprin *et al.*, 1999). Monteville *et al.* (2003) also found that a *C. jejuni cadF* mutant bound to fibronectin at levels less than 10% of the wild type *C. jejuni* F38011 and the clinical strain, 81-176, suggesting that CadF is the primary Fn-binding constituent in these two strains. Furthermore, *in vitro* binding assays have revealed that the binding of a *C. jejuni cadF* mutant to INT 407 cells is reduced by 59% when compared with a wild-type isolate (Monteville *et al.*, 2003). The variation in function of the CadF protein was investigated by comparing *C. jejuni* and *C. coli* strains for their ability to attach to intestinal epithelial cells *in vitro*. *C. jejuni* strains adhered to and invaded the epithelial cells at significantly higher levels than *C. coli* strains (Hu & Kopecko, 1999; Bacon *et al.*, 2000; Biswas *et al.*, 2000; Monteville *et al.*, 2003; Nadeau *et al.*, 2003; Konkel *et al.*, 2004; Hu *et al.*, 2005). Interestingly, Konkel *et al.* (2005) have identified of a fibronectin-binding domain within the *Campylobacter jejuni* CadF protein and showed that maximal fibronectin-binding activity was localised within a 4 amino acids segment (amino acids 134–137) consisting of a phenylalanine–arginine–leucine–serine motif. The CadF protein sequence in *Campylobacter coli* contains this 4 amino acids motif, however, this motif is not present in the *A. butzleri* CadF protein.

The present study has found that the *Arcobacter* strains tested, with the exception of *A. skirrowii*, showed the diffuse pattern in which bacteria adhere evenly to the whole cell surface (Figure 3.22). Only *Arcobacter skirrowii* 449/80 strain showed a localised adherence in which bacteria formed characteristic micro-colonies on the surface of the INT-407 cell (Section 3.6). Further investigation is necessary to confirm the adherence patterns for each *Arcobacter* species and to elucidate the nature of individual adhesion mechanisms for *Arcobacter* species, particularly those that cause disease in both

humans and animals. Additional studies need to be focussed toward the identification and characterization of the CadF fibronectin-binding domains of *Arcobacter* clinical isolates. Moreover, the humoral and cellular immune responses of the host against the CadF protein should be assessed for this organism.

A series of putative virulence genes *cadF*, *ciaB*, *flaA*, *flaB* and *pldA* are recognised in *A. butzleri* strain ATCC49616. Slot blot analysis was performed (Section 3.7) to determine whether homologs of these genes were also present in *A. cryaerophilus* strain A169/B, *A. skirrowii* strain 449/80 and the two *Arcobacter* water isolates SW-DL2 and SW-OL2. Figure 3.24 shows that these genes are present in each of the strains tested. These results have been extended by a recent study (Doudah *et al.*, 2012) that has revealed the presence of homologs of nine putative *Campylobacter* virulence genes (*cadF*, *ciaB*, *cj1349*, *hecA*, *hecB*, *irgA*, *mviN*, *pldA*, and *tlyA*) in each of the three *Arcobacter* type strains tested (*A. butzleri* LMG 10828, *A. cryaerophilus* LMG 10210 and *A. skirrowii* LMG 6621;). A larger set of human and animal *Arcobacter* strains has been shown to have a variable complement of the nine genes although not every clinical *Arcobacter* isolate carried all nine of the putative genes. This is a very interesting result that might indicate different pathogenic behaviours between strains due to a greater diversity in their genomic characteristics under various environmental conditions (Ho *et al.*, 2006a; Doudah *et al.*, 2012).

The five putative virulence determinants identified in the reference and water isolates (Section 3.7) include the *cadF* gene, which has been detected in *Campylobacter* species (57% identity with the putative *Arcobacter* gene) and *Wolinella succinogenes* (59%). CadF in these species encodes an outer membrane protein that adheres to fibronectin of intestinal epithelial cells (Miller *et al.*, 2007; Konkel *et al.*, 2005; Monteville *et al.*, 2003). A homolog of the *ciaB* gene (*Campylobacter* invasive antigen B) has also been identified in various organisms, including *Campylobacter* species (59%), *Sulfurovum* species (65%), *Nitratiruptor* species (65%), *Wolinella succinogenes* (62%), and *Caminibacter mediatlanticus* (62%); these genes are involved in host cell invasion in these species (Miller *et al.*, 2007; Konkel *et al.*, 1999b). The *pldA* gene encodes an outer membrane phospholipase A, which hydrolyses acyl ester bonds. The *A. butzleri* RM4018 *pldA* gene sequence has similarity to *Campylobacter curvus* (60%),

Campylobacter concisus (58%), and to *Campylobacter fetus* subsp. *fetus* (Istivan *et al.*, 2006; Schmiel *et al.*, 1999).

In the present study, semi-quantitative measurements of *cadF* mRNA levels by slot blot hybridization showed that mRNA related to the *cadF* gene was detected in all strains. However, the band intensity (Figure 3.25) suggests that *A. cryaerophilus* A169/B and SW-OL2 showed higher expression than other strains. In addition, in RT-PCR analysis expression of *cadF* and the other putative virulence genes (*ciaB*, *pldA*, *flaA* and *flaB*) was shown to be upregulated compared with the house-keeping gene (*glnA*), in all strains used in this study. There were differences in the levels of expression of each of the mRNAs in each of the strains (Figure 3.29). The *cadF* gene was expressed at a higher level in *A. cryaerophilus* strain A169/B and *A. skirrowii* strain 449/80 when compared to the three other strains (D2686, SW-DL2 and SW-OL2) during invasion of INT-407 cells. Therefore, the analysis of relative gene expression data showed that the CadF protein may be an important virulence factor, contributing to adhesion of epithelial cells for all those species of *Arcobacter* and therefore to disease induction. It has also been shown that the four other putative genes (*ciaB*, *flaA*, *flaB* and *pldA*) showed differences in transcript levels across the strains tested (Figure 3.29). For instance, the *pldA* gene was poorly expressed in SW-DL2 although it was expressed at a higher level in other strains. Furthermore, a flagellar gene (*flaA*) was expressed at higher levels in *A. butzleri* strain D2686 and SW-DL2 than the other three strains (*A. cryaerophilus* strain A169/B, *A. skirrowii* 449/80 and SW-OL2; Figure 3.29).

Most commonly, studies on arcobacters have focused on the taxonomy and isolation of members of this genus from environmental and clinical samples, while their pathogenicity still remains poorly understood. Risk assessment strategies between *Arcobacter* species and human disease have not yet been developed, although public health concerns have been highlighted by different international health organisations. The current status of measures aimed at reducing or eradicating *Arcobacter* from the human food chain should be reviewed. Several studies have revealed that faecal shedding in livestock and wild animals is strongly correlated with food and water contamination (Stanley *et al.*, 1998; Bonardi *et al.*, 2001; Beach *et al.*, 2002). Therefore, it is essential to reduce contamination by these bacteria through the implementation of

preventive measures for food safety and during the treatment of drinking waters. The pathogenic role and potential virulence factors of arcobacters have to be further examined. Adhesion of bacteria to mucosal surfaces is necessary for colonisation and is a critical step in pathogenicity, particularly in cases where the pathogen is confined to a mucosal surface such as the respiratory, urinary, and gastrointestinal tracts of humans and animals. Understanding the roles of the adhesins and the associated signal transduction pathways, coupled with an effective approach to risk management across the food and water supply chain, will provide routes to developing control strategies for these organisms.

Conclusion

1. Only two out of eleven surface water sources were contaminated with *Arcobacter* (18%). This is the first reported isolation of *Arcobacter* spp. in Scotland.
2. Two water *Arcobacter* isolates (SW-DL2 and SW-OL2) were obtained from two Scottish surface waters (Duddingston Loch and Loch Ore).
3. The three reference strains (*Arcobacter butzleri* D2686, *Arcobacter cryaerophilus* A169/B, and *Arcobacter skirrowii* 449/80) and the two water *Arcobacter* isolates (SW-DL2 and SW-OL2) degraded elastin and gelatin.
4. The ECPs of all strains showed positive gelatinase activity (50-310 units).
5. The ECP of all strains caused haemolysis of two classes of erythrocytes (from sheep and chicken). *A. butzleri* D2686 also showed poor haemolysis of human erythrocytes.
6. The *cadF* gene was cloned and sequenced from *A. butzleri* D2686 and SW-OL2 and the *cadF* gene sequence of SW-OL2 showed high level of sequence identity (96%) with a gene that encodes a Fnb-like protein in *A. butzleri* RM4018 (the type strain of the species).
7. All the strains were able to adhere to INT-407 cells *in vitro*. The highest percentage (81%) was observed for *A. butzleri* strain D2686 isolated from a human diarrhoea sample.
8. In this study, the *cadF* knockout mutants showed a significant reduction in adherence for *A. butzleri* D2686- $\Delta cadF$ ($P > 0.01$) and SW-OL2- $\Delta cadF$ ($P > 0.001$) mutant strains.

9. Five putative virulence genes (*cadF*, *ciaB*, *flaA*, *flaB* and *pldA*) have been identified by slot blot hybridization in all strains used in this study (*A. butzleri* D2686, *A. cryaerophilus* A169/B, *A. skirrowii* 449/80, SW-DL2 and SW-OL2).
10. Semi-quantitative measurements of *cadF* mRNA levels by slot blotting showed that mRNA related to the *cadF* gene was present in all strains; however, *A. cryaerophilus* A169/B and SW-OL2 gave a slightly stronger signal than other strains.
11. RT-PCR analysis revealed that five putative virulence genes (*cadF*, *ciaB*, *flaA*, *flaB* and *pldA*) were upregulated in all strains on infection of tissue culture cells in this study with a significant difference of expression levels; the *cadF* gene was expressed at the highest level in *A. cryaerophilus* strain A169/B.

Future Directions

The increasing prevalence of *Arcobacter* species worldwide has prompted a significant amount of research that has focussed on *Arcobacter* isolation and taxonomy in the last decade. However, a better understanding of their pathogenicity determinants is still required. There are many possible avenues of investigation and future work will focus on examining the interactions of the putative *Arcobacter* adhesins with eukaryotic cells through the integral components of focal adhesions. Further investigation is also necessary to confirm the role of CadF proteins and will require the purification of CadF variant proteins from pathogenic *Arcobacter* species and the identification of fibronectin-binding domains of CadF proteins.

The host immune system is also important in the disease outcome of these bacteria and studies are necessary to help drive the development of immune-based strategies that may be used to control infection in animal hosts. Identification of other putative binding factors in *Arcobacter butzleri* is needed to permit the construction of attenuated vaccine strains and detection of bacterial receptors may provide new molecular therapeutic targets against *Arcobacter* species infection.

In conclusion, considerable further investigation is necessary to identify novel virulence factors and to define the function of known virulence associated genes which have already been detected within the genome of *Arcobacter butzleri* strain RM4018.

APPENDICES

Appendix 1. Gene sequences and alignments

1. Partial sequence of 16S rDNA gene of SW-DL2 (water *Arcobacter* isolate from this project)

```
TCGTCCATCATCACTGACGGGAGGTCGCTACTCATAGGACACTACACTC
ACATTCGAATGAGTTCAACTCCCATGGTGTGACGGGCGGTGAGTACAAG
ACCCGGGAACGTATTCACCGTAGCATAGCTGATCTACGATTACTAGCGA
TTCCAAC TTCATGTAGTCGAGTTGCAGACTACAATCCGAAC TGGGAGGC
ATTTTTGAGATTTGCTCCACGTCACCGTATTGCTGCTCTTTGTATACCC
CATTGTAGCACGTGTGTAGCCCTGGACGTAAGGGCCATGATGACTTGAC
GTCATCCTCACCTTCCTCCTACTTGCGTAGGCAGTCTCCTTAGAGTTCT
CAGCCGAAC TGTAGCAACTAAGGACGAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATCTCACGACACGAGCTGACGACAGCCGTGCAGCACCTG
TATGCAAGTTTCTGCAAGCAGACACTAATCTATCTCTAAATCATTCTTA
CTATGTCAAGTCCAGGTAAGGTTCTTCGTGTATCGTCGAATTAAACCAC
ATGCTCCACCGCTTGTGCGGGTCCCCGTCTATTCCTTTGAGTTTTAATC
TTGCGACCGTACTCCCCAGGCGGTACACTTAATGTGTAACTGCATTAC
TGCAAGGTCGAGCCTCACAACAAC TAGTGTACATCGTTTAGGGCGTGGA
CTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGTCTCAGC
GTCAATATTGTTCCAGTAGATCGCCTTCGCAATCGGTATTGCATCTGAT
CTCTACGGATTTTACCCCTACACCAGAAGTTCCATCTACCTCTCCCACA
TTCTAGAGTACAGGTTTCAAAGCAGTCTATAGTTAGGCTATAGGATGTC
ACTTCAACTATCAGTCGCTACGCGCTCTTTACGCCAGTGAATACGAGTA
CGCTGCACCCTCGTATACAGCGCTGCTGGCACGGAGTAGCGTGCTAATC
ATATATACGTCATACTCTATTAACGAGTACGCACGTATGGTCTCTCAGC
AGCGTGCTGCACGACTCGTCATGTGCATATCCGATGTTGCTCGAGACTG
ACGAGAGTCAGTCAGTGCAGTATCTCTCACTTAGCGTCATGCTGTAGGC
TAGCGCACTGCATTAGCAGCGTACAAC TTCCGTTACGTG
```

2. Alignment of sequences of the 16S rDNA genes of SW-DL2 and *A. butzleri* RM4018

Score	Identities	Gaps
1583 bits (857)	928/959 (97%)	18/959 (1%)
Query-53 TTCGAATGAGTTCAACTCCCATGGTGTGACGGGCGGTGAGTACAAGACCCGGGAACGTAT 112		
Sbjct-1412TTCGAATGAGTTCAACTCCCATGGTGTGACGGGCGGTGAGTACAAGACCCGGGAACGTAT 1353		
Query-113 TCACCGTAGCATAGCTGATCTACGATTACTAGCGATTCCAACCTCATGTAGTCGAGTTGC 172		
Sbjct-1352TCACCGTAGCATAGCTGATCTACGATTACTAGCGATTCCAACCTCATGTAGTCGAGTTGC 1293		
Query-173 AGACTACAATCCGAACCTGGGAGGCATTTTGTGAGATTGCTCCACGTACCGTATTGCTGC 232		
Sbjct-1292AGACTACAATCCGAACCTGGGAGGCATTTTGTGAGATTGCTCCACGTACCGTATTGCTGC 1233		
Query-233 TCTTTGTATACCCATTGTAGCACGTGTGTAGCCCTGGACGTAAGGGCCATGATGACTTG 292		
Sbjct-1232TCTTTGTATACCCATTGTAGCACGTGTGTAGCCCTGGACGTAAGGGCCATGATGACTTG 1173		
Query-293 ACGTCATCCTCACCTTCTCTACTTGCCTAGGCAGTCTCCTTAGAGTTCTCAGCCGAAC 352		
Sbjct-1172ACGTCATCCTCACCTTCTCTACTTGCCTAGGCAGTCTCCTTAGAGTTCTCAGCCGAAC 1113		
Query-353 TGTTAGCAACTAAGGACGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAC 412		
Sbjct-1112TGTTAGCAACTAAGGACGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAC 1053		
Query-413 ACGAGCTGACGACAGCCGTGCAGCACCTGTATGCAAGTTTCTGCAAGCAGACACTAATCT 472		
Sbjct-1052ACGAGCTGACGACAGCCGTGCAGCACCTGTATGCAAGTTTCTGCAAGCAGACACTAATCT 993		
Query-473 ATCTCTAAATCATTCTTACTATGTCAAGTCCAGGTAAGGTTCTTCGTGTATCGTCGAATT 532		
Sbjct-992 ATCTCTAAATCATTCTTACTATGTCAAGTCCAGGTAAGGTTCTTCGTGTATCGTCGAATT 933		
Query-533 AAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCTATTCTTTGAGTTTAAATCTTGC 592		
Sbjct-932 AAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCTATTCTTTGAGTTTAAATCTTGC 873		
Query-593 GACCGTACTCCCCAGGCGGTACACTTAATGTGTTAACTGCATTACTGCAAGGTCGAGCCT 652		
Sbjct-872 GACCGTACTCCCCAGGCGGTACACTTAATGTGTTAACTGCATTACTGCAAGGTCGAGCCT 813		
Query-653 CACAACAAC TAGTGATCATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC 712		
Sbjct-812 CACAACAAC TAGTGATCATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC 753		
Query-713 TCCCCACGCTTTCGCGTCTCAGCGTCAATATTGTTCCAGTAGATCGCCTTCGCAATCGGT 772		
Sbjct-752 TCCCCACGCTTTCGCGTCTCAGCGTCAATATTGTTCCAGTAGATCGCCTTCGCAATCGGT 693		
Query-773 ATTGCATCTGATCTCTACGGATTTTACCCCTACACCAGAAGTTCCATCTACCTCTCCAC 832		
Sbjct-692 ATTCTTCTGATCTCTACGGATTTTACCCCTACACCAGAAATTCATCTACCTCTCCAC 633		
Query-833 ATTCTAGAGTA-CAGGTTTCAAAGCAGT-CTATAGTTAGGCTATAGGATGTCACTTCAA- 889		
Sbjct-632 ATTCTAGATTAAACAGTTTCAAAGCAGTTCTATAGTTAAGCTATAGGATTTCACTTCAAA 573		
Query-890 CT-ATCAGTC-GC-TACGCGCTCTTTACGCC-AGTGAATACGAGTA-CGCT-GCACCTC 943		
Sbjct-572 CTTATCAATCCGCCTACACGCTCTTTACGCCAGTGATTCCGAGTAACGCTTGCACCTC 513		


```
Query-944 -GTAT-ACAGCG-CTGCTGGCACGGAGT-AGC-G-TGCTAAT-CATATA-TAC-GTCAT 993
          |||| || ||| |||||||||||||||| ||| | |||| || |||||| ||| |||||
Sbjct-512 CGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTATTCATATAATACCGTCAT 454
```

Query: 16S rDNA gene of SW-DL2

Sbjct: 16S rDNA gene of *Arcobacter butzleri* RM4018 strain RM4018

3. Partial sequence of 16S rDNA gene of SW-OL2 (water *Arcobacter* isolate from this project)

```
TTGAGTCATTCATCACTGACGGGAGGTAGCTACTCATAGACATCCCCGC
TTCGAATGAGTTCAACTCCCATGGTGTGACGGGCGGTGAGTACAAGACC
CGGGAACGTATTCAACCGTAGCATAGCTGATCTACGATTACTAGCGATT
CAACTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTGGGAGGCATT
TTTGAGATTTGCTCCACGTCACCGTATTGCTGCTCTTTGTATACCCCAT
TGTAGCACGTGTGTAGCCCTGGACGTAAGGGCCATGATGACTTGACGTC
ATCCTCACCTTCCTCCTACTTGCGTAGGCAGTCTCCTTAGAGTTCTCAG
CCGAACTGTTAACAATAAGGACGAGGGTTGCGCTCGTTGCGGGACTTA
ACCCAACATCTCACGACACTAGCTGACAACAGCCGTGCAGCACCTGTAT
GCAAGTTTCTGCAAACAGACACTAATCTATCTCTGAATCATTCTTACTA
TGTC AAGTCCACGTAAAGTTCTTCGTGTATCGTCGAATTAAACCACATG
CTCCACCGCTTGTGCGGCTCCCCGTCTATTCTTTGAGTAGTTATCTTG
CGACCGTACTCCCCACGCGGTAGAAGTAATGTGGGATCTGCGTTGACTG
CGGGGTAGAGCCTCACAACAACAAGAGTACATCGTTTATGGAGGGGGTA
CGACCAGGGGGCTTATTCATGCTTGCTCGTCACGCTGTGTGGTCGTCAA
CGACGATAATATTCCTCGTAGGATCGCCCTCCTCATAAGTAGTCTTCCTG
ATGTGGTGCGGAGTGTATTTCTACACAAAAAATACATCTAACTTTTGT
GCATCTATAAACACCTGTCTATAAAATCATAGTTAGTAACGAGTGCACT
CCACTAGGAGTAGGCTGGAGTCTACTCATCACCAGATCTCTACATGAAT
GACCACTATATGATTCGGATGAGTGTGATCGACTACTGAACAGGCTAC
GTCGCTTTCATAGAGATGGTAGGAGTAATGCGCACACGGGCTAGCTATC
ATCGTAATACAACCTCCGCTCGCTAGAAGCGAGCGTATCAGGTCTACGCA
TTCAGATGCGTAGGAACA
```

4. Alignment of sequences of the 16S rDNA genes of SW-OL2 and *A. butzleri* RM4018

Score	Identities	Gaps
1055 bits(571)	691/749(92%)	8/749(1%)

Query-14 CACTGACGGGAGGTAGCTACTCATAGACATCCCCGTTCGAATGAGTTCAACTCCCATGG 73
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct-1445CACTG-TGGAAGGTAGCTACT-TTAG-CATCCCCGTTCGAATGAGTTCAACTCCCATGG 1389

Query-74 TGTGACGGGCGGTGAGTACAAGACCCGGAACGTATTACCGTAGCATAGCTGATCTACG 133
 |||
 Sbjct-1388TGTGACGGGCGGTGAGTACAAGACCCGGAACGTATTACCGTAGCATAGCTGATCTACG 1329

Query-134 ATTACTAGCGATTCCAACCTTCATGTAGTCGAGTTGCAGACTACAATCCGAAGTGGGAGGC 193
 |||
 Sbjct-1328ATTACTAGCGATTCCAACCTTCATGTAGTCGAGTTGCAGACTACAATCCGAAGTGGGAGGC 1269

Query-194 ATTTTGGAGATTGCTCCACGTACCGTATTGCTGCTCTTTGTATACCCATTGTAGCAC 253
 |||
 Sbjct-1268ATTTTGGAGATTGCTCCACGTACCGTATTGCTGCTCTTTGTATACCCATTGTAGCAC 1209

Query-254 GTGTGTAGCCCTGGACGTAAGGGCCATGATGACTTGACGTCATCCTCACCTTCCTCCTAC 313
 |||
 Sbjct-1208GTGTGTAGCCCTGGACGTAAGGGCCATGATGACTTGACGTCATCCTCACCTTCCTCCTAC 1149

Query-314 TTGCGTAGGCAGTCTCCTTAGAGTTCTCAGCCGAAGTGTAAACAATAAGGACGAGGGTT 373
|||||
Sbjct-1148TTGCGTAGGCAGTCTCCTTAGAGTTCTCAGCCGAAGTGTAGCAACTAAGGACGAGGGTT 1089

Query-374 GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACTAGCTGACAACAGCCGTGCAGC 433
 |||
 Sbjct-1088GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACAGCTGACGACAGCCGTGCAGC 1029

Query-434 ACCTGTATGCAAGTTTCTGCAAACAGACACTAATCTATCTCTGAATCATTCTTACTATGT 493
|||||
Sbjct-1028ACCTGTATGCAAGTTTCTGCAAACAGACACTAATCTATCTCTAAATCATTCTTACTATGT 969

Query-494 CAAGTCCACGTAAAGTTCTTCGTGTATCGTCAATTAAACCACATGCTCCACCGCTGTG 553
|||||
Sbjct-968 CAAGTCCAGGTAAGGTTCTTCGTGTATCGTCAATTAAACCACATGCTCCACCGCTGTG 909

Query-554 CGGCTCCCCGTCTATTCCTTTGAGTAGTTATCTTGCACCGTACTCCCACGCGGTAGAA 613
||| ||||||| | ||||||| |||||
Sbjct-908 CGGGTCCCCGTCTATTCCTTTGAGTTTTAATCTTGCACCGTACTCCCAGGCGGTACAC 849

Query-614 GTAATGTGGGATCTGCGTTGACTGCGGGGTAGAGCCTCACAAACAAGAGTACATCGTT 673
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct-848 TTAATGTGTTAACTGCATT-ACTGCAAGGTCGAGCCTCACAACTAGTGACATCGTT 790

Query-674	TATGGAGGGGTACGACCAGGGGGCTTATTCATGCTTGCTCGTCACGCTGTGTGGTCGTC	733
Sbjct-789	TAGGGCGTGG--ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGTC-TC	733

Query-734	AACGACGATAATATTCCCGTAGGATCGCC	762
Sbjct-732	AGCGTCAATATTGTTCCAGTAG-ATCGCC	705

Query: 16S rDNA gene of SW-OL2

Sbjct: 16S rDNA gene of *Arcobacter butzleri* RM4018

5. Sequence of the *cadF* gene of *Arcobacter butzleri* strain RM4018

CadF3 Fwd primer

TCTGCAATAATAGGAGAAGCAATTAGACGAATTCATAATAATGAATCTGT
TAATTCTATTTTAAACAATTAACATATTCGTTAATATTCTTCTAATAAAAA
TTATCATAAAATTATTATTAGAAATAAAATTTTAAAGAAAGGAAA

CadF-FP1 primer

GTTTATGAAAAAAGTATTATTATCAACAATTGCTTGTGCTTCTTTAGCA
TTAGCTGCAAATAGTGATTACAAATATGAAATTACTCCATTAATCGGTG

CadF- Fwd primer

GAGTTTTAACTGAAGGAAATACAGGTTTAGAGAAAACTATGCTAACGCT
GGTTTATCTTTTGATTTAATCAATTTGATTCAATTTATCGATCAAGTA

CadF-Rev* primer

GAATTAGGGTTTTTAAGAACTCTTGAAGACGTTGATGGAAAAGGAACTT
TAGTAATAGAGATACAGGAGTAACAAGAGTATTTGCTAACTTAGTAAAAG
ACTATGATTTAACTTCTGATTTATCTTTATATACATTAGTTGGAGCTGGA
GTTGAGTTCTTTGATAATGAATTTGAAGACAATAAAATGGTTTATTTGG
TAACTACGGTGTAGGAGTAAAATATAATTTAGCTGAAAGACTAGCTTTAA
AATTCGATGTTAGACATTTAATTGAAGTTGATCATGGAGATAATACATTA
TTATACACAGTAGGATTATCAGTTCCATTTGGAGAAGTTTCAAACCCAGC
TCCAGTTGCTGAAAAACCAGCTCCAGTTGCTACTCCAGTTGCTGCTCCAA
AAGATTCTGATGGTGATGGTGTTATTGATAATTTAGATGAATGCCCAAAT
ACAATGAAAGGTGCAAAAGTTGACAATATTGGTTGTATGACTTTAGTTAA
CTTAAATATCAACTTCGATACTGATAAATCAGTAATTAAAGACTCTTACA
ATTCAAGAATTAATGAATTTGCAAAAGTTATGAAAGCTGATCCAAAATTA
AAAGCAAACATTGAAGCTCATACAGATTCAGTTGGAACAGATGCTTATAA
TCAAAAATTATCAGAAAGAAGAGCTACATCTGCTGTTAATGCTTTAGTTG
CAGCTGGTGTTGAAAAAGATAGAATTAAAGCTGTTGGATATGGTGAGTCT
AGACCAATCGCTTCTAATGATACAGTTGAAGGTA

CadF-RP1* primer

GAGCTGAAACAGAAGAGTTGAAGCTGTAATGGTTAAATAAATTTAACTGT
TTTTCTTTTTTAAAGGGTTAGAAAATATTTTCTAACCCCTTTTTTATTGCC
TAATTTTTTATTGATAACTTCGTATCCAGCATCTACTATTACATCAG

CadF3 Rev* primer

ATAAAGTTTGTTCTGATATATCTTTTGACATTTTAACTGTAACATTT

Bright Green: Start code

Red: Stop code

Pink: Primers for RT-PCR and Dig-labelling

Yellow: Amplification primers for gene cloning

(*): The primer sequence is the reverse complement of this gene sequence

Appendix 2. Buffers and solutions

***Arcobacter* Broth**

- 18.0 g/l Peptone
- 1.0 g/l Yeast extract
- 5.0 g/l Sodium chloride

Coomassie destain solution

- 40% (v/v) Methanol
- 10% (v/v) Acetic acid
- 50% (v/v) Distilled water

DIG-1 Buffer

- 0.1M Tris-HCl pH 8.5, 1M NaCl, 0.2% Tween-80

DIG-4 Buffer

- 0.1M Tris-HCl pH 9.5, 0.1M NaCl, 0.2% Tween-80

Hybridization buffer

- 6M Urea
- 6x SSC (from 20x SSC)
- 1% SDS
- 50mM Tris (pH 7.5)

LB (Luria broth or Lysogeny broth)

- 10 g/l Tryptone
- 5 g/l Yeast extract
- 10 g/l NaCl

PEG solution

- 10 g Polyethylene glycol 8000 (20% PEG) and 7.3 g NaCl (2.5 M NaCl)

SAP buffer (10X)

- 0.1 M Tris-HCl (pH 7.5 at 37°C), 0.1 M MgCl₂ and 1 mg/ml BSA

SDS-PAGE resolving gel in 10-15 ml

	12%	10%
• Separating buffer (1.5M Tris-HCl, pH8.7)		
• Distilled water	7.0 ml	9,5 ml
• 30% Arcylamide/Bis acrylamide	8.0 ml	5 ml
• 10% SDS	200 µl	200 µl
• 10% APS	100 µl	100 µl
• TEMED	20 µl	20 µl

SDS-PAGE stacking gel 4% in 10 ml

- 2.5 ml Stacking buffer (0.5M Tris-HCl, pH6.8)
- 6.1 ml Distilled water
- 1.34 ml Arcylamide/bisacrylamide
- 100 µl 10% SDS
- 50 µl 10% APS
- 20 µl TEMED

SOC medium

- 2% (w/v) tryptone
- 0.5% (w/w) yeast extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM MgCl₂
- 10 mM MgSO₄
- 20 mM glucose.

20X SSC

- 3M NaCl, 0.3M Sodium acetate (pH-7.2) with DEPEC treated water

2X SSC

- SSC from 20X SSC with 0.1% SDS

0.2X SSC

- SSC from 20XSSC with 0.1% SDS

50×TAE buffer

- 242g Tris Base (MW=121.1)
- 57.1 ml Glacial Acetic Acid, 100 ml
- 0.5 M EDTA add ddH₂O up to 1000ml

1×TAE buffer (Final working concentration)

- 0.4 M Tris-acetate, 0.001 M EDTA

TE buffer

- 10 mM Tris-Cl, pH 7.5
- 1 mM EDTA

5X TdT buffer

- 1M potassium cacodylate
- 125 mM Tris
- 0.05% (v/v) Triton X-100
- 5 mM CoCl_2 (pH 7.2).

Transformation buffer

- 10 mM Pipes-HCl, pH 6.7
- 15 mM CaCl_2
- 250 mM KCl
- 55 mM MnCl_2

Tris-glycine running buffer 10X

- 30 g Tris base
- 144 g Glycine
- 100 ml (10%) SDS
- dH_2O to 1 Litre pH to 8.3 with HCl.

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